JOURNAL OF AGRICULTURAL RESEARCH

VOL. 64 WASHINGTON, D. C., APRIL 15, 1942 NO. 8

NATURAL MODE OF ENTRANCE OF FUNGI INTO CORN EARS AND SOME SYMPTOMS THAT INDICATE INFECTION [1]

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INTRODUCTION

Very little has been published regarding the time and manner in which fungi enter and permeate developing ears of corn (*Zea mays* L.). Since no very satisfactory way of controlling the corn ear rots is known, it seemed desirable to learn more about the actual course or path of infection. Such a study was started in the season of 1933 and continued during the seasons of 1935 and 1937. The work was suspended during 1934 because of severe chinch bug damage to the corn on the station farm, and little was accomplished in 1936 because of damage from drought.

TECHNIQUE

METHOD 1

The principal procedure was the plating of natural unsterilized pieces of tissue from different parts of the ear. The station strain of Reid Yellow Dent open-pollinated corn was used. Work was limited to ears that were 7 or more inches in length, were enclosed tightly by the husks, were not penetrated farther than the tip end by corn earworms (*Heliothis armigera* (Hbn.)), and did not already show visible signs of moldiness except sometimes a slight amount at the tip end. Otherwise, no selection of ears was made. Ears that were slightly exposed to the weather at the tip end only, or showed signs of corn earworm activity at the tip of the ear, with or without accompanying mold growth, were classified and handled separately from the ears that were well covered with husks and appeared entirely sound.

Ears were broken off with shank and husks intact at different stages, ranging from late-milk, average grain moisture 55.6 percent, to mature average moisture 24.9 percent (table 1). The outer husks were removed out of doors, leaving two or three layers of the clean inner husks to cover the kernels. The tips of the husks were then snipped off even with the tips of the ears and the ears were placed tip down in a large clean pail containing a 1:200 formalin solution 1 inch deep at the bottom. This process killed any mold at the tips of the ears and thus helped to prevent contaminations. The pail with corn ears was then covered with clean wet toweling and brought into the laboratory.

[1] Received for publication July 7, 1941.

TABLE 1.—*Fusarium moniliforme infection on or in kernels of Reid Yellow Dent corn at different times during kernel development, studied by method 1, 3-year average, Urbana, Ill., 1933, 1935, and 1937*

Date	Average moisture content of—		Condition of tip of ear	Ears in test	Ears with infected kernels—	
	Grain	Cob			Without surface sterilization	After surface sterilization
	Percent	*Percent*		*Number*	*Percent*	*Percent*
Sept. 2–5	55.6	60.2	Covered and sound	190	3.2	0.0
			Exposed or injured	102	18.6	.0
Sept. 13–15	46.5	58.3	Covered and sound	216	17.1	.0
			Exposed or injured	97	52.6	5.2
Sept. 24–25	33.6	56.0	Covered and sound	156	27.6	.0
			Exposed or injured	105	60.0	8.6
Oct. 5–7	29.1	50.0	Covered and sound	135	33.3	3.0
			Exposed or injured	66	69.7	9.1
Oct. 15–18	24.9	40.2	Covered and sound	113	40.7	4.4
			Exposed or injured	60	71.6	28.3

Each ear was broken in the middle with a specially designed apparatus. While the cob was being broken the husk generally remained intact. In the plating room, the husks were opened, short pieces of silk were snipped with sterile instruments from around the central section of the ear, and laid on potato-dextrose agar in Petri dishes (fig. 1, *A*, *d*). Then kernels were taken off with pedicels attached (fig. 2, *A*) and the pedicel was torn off and laid after the corresponding kernel clockwise in the Petri dish, five from each ear (fig. 1, *A*, *a*, *b*). Several fragments from the vascular cylinder were placed in the center of the dish (fig. 1, *A*, *c*). Then the shank was broken off from the ear and a fragment from the center of the butt of the cob was plated (fig. 1, *A*, *e*). A total of 1,240 ears were investigated in this manner.

The broken pieces of ears were immediately dried by forced warm air and later internal seed infection was determined by taking five kernels from the broken part of the ear, neighbors to the kernels that had been plated earlier (fig. 2, *A*). These were then surface-sterilized for 90 minutes in a 4-percent solution of calcium hypochlorite and plated.

METHOD 2

Additional data on the effect of slight earworm damage on kernel infections was obtained in another series of experiments conducted in 1933–37 with five kinds of corn (table 2). Well-matured seed ears with good husk covering were harvested in the usual manner about the middle of October and were rack-dried. Three classifications were made: (1) Ears entirely sound and bright; (2) Ears slightly injured by earworms at the tip only, with no mold growth apparent to the naked eye; and (3) Ears slightly injured by earworms and molded, usually by *Fusarium moniliforme*. Ears with injuries more extensive than those shown in figure 3 or with moldy kernels at other locations than at the tip end were discarded.

The selected ears, 1,462 in all, were tipped and butted by removing 2 or more inches from the tip and about 1 inch from the butt. Care

FIGURE 1.—Unsterilized tissues of corn ears plated aseptically on potato-dextrose agar and incubated for 4 days: *A*, All parts of the ear used in plating, sterile; *B*, *Fusarium moniliforme* in silks and on parts of kernels that were in contact with silks; *C*, *F. moniliforme* has penetrated to all parts plated except the butt end of the cob. *a*, Kernel; *b*, pedicel removed from kernel *a*; *c*, fragments removed from vascular cylinder of cob; *d*, short sections from silks; *e*, fragment removed from end of cob where shank had been broken off; *f*, *Monilia sp*. Parts shown in *a-d* were taken from nearly the same plane across the center of the ear; all fungus colonies shown except *f* are *F. moniliforme*.

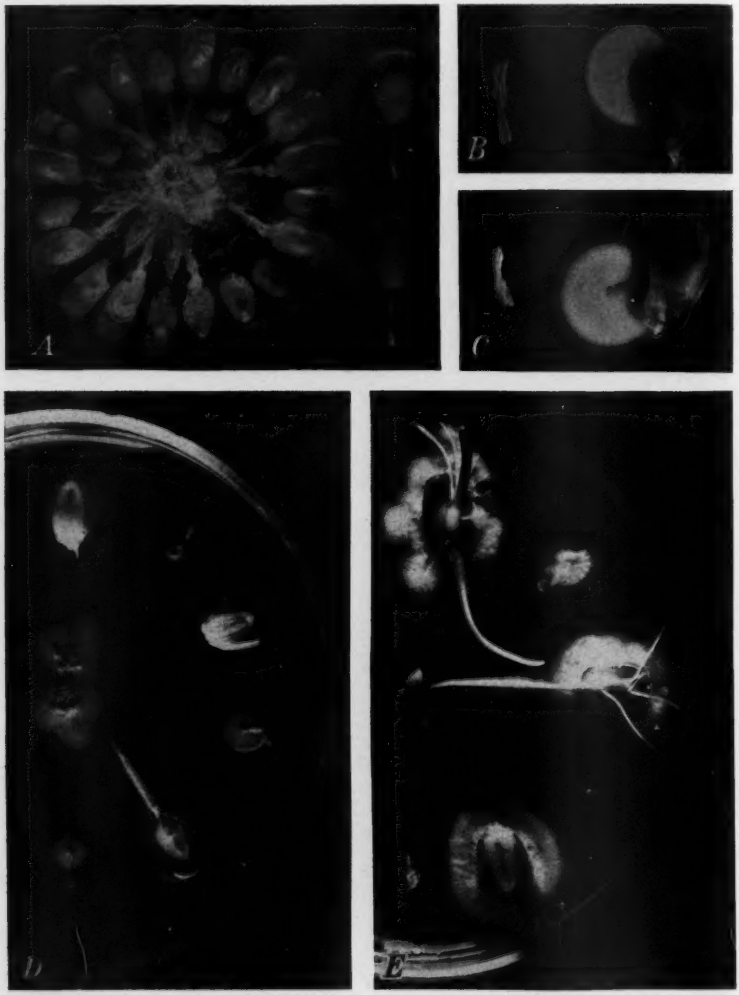

FIGURE 2.—*A*, A broken ear of corn showing how the kernels may be removed from the cob with their pedicels attached; *B*, a colony of *Cephalosporium acremonium* which originated at the point where the kernel had been in contact with the silk; *C*, a colony of *C. acremonium* that originated from a glume; *D*, *C. acremonium* infection in the vascular cylinder and the lower end of pedicels only; *E*, *C. acremonium* infection on kernels only and the distal end of one pedicel. *B* and *C* were photographed from the reverse aside of the agar plates. The progression of infection in *D* and in *E* is in opposite directions.

was taken to go at least 1 inch beyond the worm-injured or moldy area. Four kernels were then removed from each end of the remaining part of each ear, surface-sterilized, and plated on potato-dextrose agar. After 5 days identifications of fungus colonies originating from infections within the kernels were made.

METHOD 3

Mature, well-developed ears of the station strain of Reid Yellow Dent corn were tested for the presence and purity of several kinds

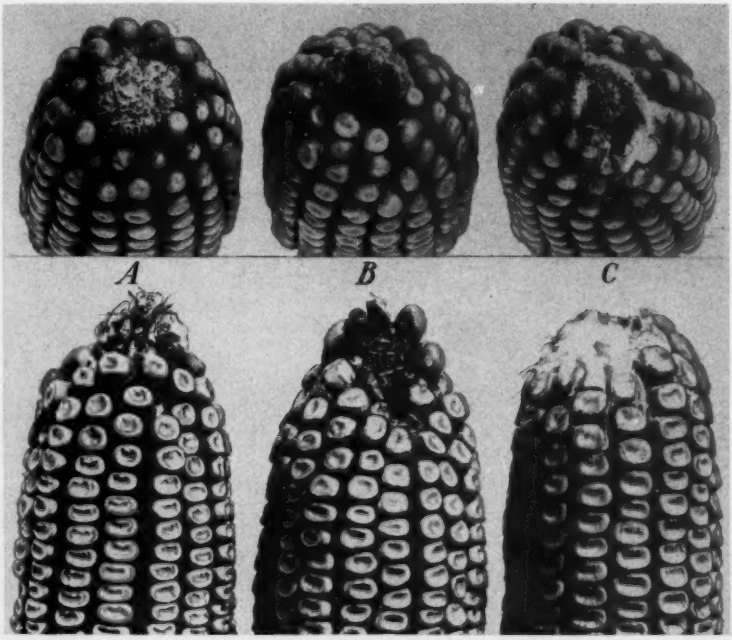

FIGURE 3.—Tip ends of corn ears: *A*, Free from discolorations, worm injury, or moldiness; *B*, slightly damaged by earworms at the tip only; *C*, slightly damaged by earworms, followed by moldiness. These ears are typical of the classes given in table 2.

of fungus infections by removing representative kernels, surface-sterilizing, and plating. From this group two kinds of ears were selected for study: (1) Those that tested pure for one kind of infection but showed no external symptoms on the kernels, and (2) those that tested pure for one kind of infection and showed slight outward discoloration on the kernels from infection.

Five kernels from each ear were then soaked in tap water overnight, sterilized for 1 minute in 1:1,000 $HgCl_2$, washed in three changes of sterile water, and dissected. After the first section was made (fig. 5, *A*) each part was dissected separately with freshly sterilized instruments to avoid carrying the fungus from one part of the kernel to another.

TABLE 2.—*Ear-tip injury by corn earworms in relation to internal fungus infection of the kernels as determined by surface-sterilizing and plating four kernels from the tip half and four from the butt half of each ear, all the kernels being sound in appearance; studied by method 2 in experiments conducted between 1933 and 1937, inclusive*

Year	Variety or hybrid	Ear-tip condition	Ears tested	Ears with kernel infection at tip or butt half									
				Fusarium moniliforme		*Cephalosporium acremonium*		*Diplodia zeae*		*Nigrospora* species		*Gibberella zeae*	
				Tip	Butt	Tip	Butt	Tip	Butt	Tip	Butt	Tip	Butt
			Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1933	Reid Yellow Dent	Sound and bright	181	19.9	11.0	2.8	2.8	2.2	13.2	0.0	0.0	0.6	0.0
		Slightly injured, no mold	68	45.6	25.0	.0	1.5	2.9	7.4	.0	.0	.0	.0
		Slightly injured, moldy	76	78.9	48.7	.0	.0	2.6	7.9	.0	.0	1.3	.0
1935	Golden Beauty	Sound and bright	200	4.5	2.0	1.0	.5	.0	.0	.0	.0	.5	.0
		Slightly injured, no mold	80	12.5	6.3	1.3	2.5	.0	.0	1.3	.0	.0	.0
		Slightly injured, moldy	67	37.3	14.9	6.0	3.0	3.0	3.0	3.0	4.5	3.0	.0
1935	Illinois Hybrid 39	Sound and bright	50	6.0	4.0	2.0	4.0	2.0	2.0	.0	.0	4.0	.0
		Slightly injured, no mold	50	12.0	10.0	4.0	4.0	4.0	2.0	.0	.0	9.0	.0
		Slightly injured, moldy	20	60.0	45.0	10.0	5.0	.0	.0	.0	.0	.0	.0
1936	Illinois Hybrid 172	Sound and bright	62	17.7	9.7	1.6	.0	3.2	4.8	.0	.0	3.2	.0
		Slightly injured, no mold	70	22.8	12.9	.0	2.9	4.3	2.9	.0	.0	5.7	.0
		Slightly injured, moldy	39	56.4	33.3	.0	.0	2.6	2.6	.0	.0	18.0	.0
1937	Illinois Hybrid 960	Sound and bright	230	2.6	1.7	2.2	1.7	.4	.4	7.0	3.5	.4	.0
		Slightly injured, no mold	187	6.4	3.7	5.9	3.7	1.6	1.1	7.0	5.3	1.6	.0
		Slightly injured, moldy	82	69.5	40.2	2.4	2.4	.0	.0	8.5	8.5	6.1	1.2
	Weighted average	Sound and bright	723	9.0	5.0	2.0	1.7	1.1	4.0	2.2	1.1	1.0	.0
		Slightly injured, no mold	455	16.5	9.4	3.1	3.1	2.2	2.2	3.1	2.2	2.5	.0
		Slightly injured, moldy	284	62.0	35.9	2.8	1.8	1.8	3.2	3.2	3.5	5.3	.3

Kernels that showed white stripes on the surface of the pericarp were studied also by means of free-hand and paraffin-embedded sections.

SPECIES OF FUNGI INVADING CORN EARS

USE OF THE WORD "INFECTION"

For simplicity in presentation, the presence of fungi in any part of the ear described in the first column of table 3 will be referred to as "infection." Strictly speaking, some of this may be only contamination. The fungus entering the ear must obtain food and it is only a question whether the food is obtained from dead or live tissue. The silks are the first parts of an ear that might be considered as dying, but actually as long as the husks are green and tight the unexposed parts of the silks also appear to be alive even though they have already served their function. In healthy ears they are turgid during this period and a bright pale-green, tan, or brownish color, depending on the genetic complex. The fact that only some kinds of fungi were found on or in the silks around the middle of the ear beneath the husks of immature ears makes the propriety of the use of the word "contamination" doubtful in most cases. Not until the husks dry does the silk covered by the husks dry and shrivel, and, except for earworms, it was not until this time that some ears were found with small insects in them, or water beneath the husks.

FUSARIUM MONILIFORME INFECTION

More ears were entered by *Fusarium moniliforme* Sheldon than by any other fungus. This was true even when only ears well covered by husks and free from injury by worms or birds are considered. In the parts of the ear that were well covered by husks, infection was found in or on the silks more often than on the kernels directly beneath the silks (table 3). Next in decreasing order were kernels, pedicels, vascular cylinder, and butt of cob. No doubt infection moved in the direction of decreasing prevalence. With rare exceptions, infection either involved the silks only, or silks plus kernels (fig. 1, *B*), or silks plus kernels plus pedicels, or silks plus kernels plus pedicels plus vascular cylinder (fig. 1, *C*), or silks plus kernels plus pedicels plus vascular cylinder plus butt of cob.

TABLE 3.—*Reid Yellow Dent corn ears infected with Fusarium moniliforme or Cephalosporium acremonium in various locations in the ears, as determined by removing the tissues aseptically and plating on agar medium: Based on 1,240 ears used at Urbana, Ill., in 1933, 1935, and 1937*

[No disinfection used on the parts plated; studied by method 1]

Location of infection in corn ear	Infected with *Fusarium moniliforme*		Infected with *Cephalosporium acremonium*	
	Ears with covered, sound tips	Ears with exposed or injured tips	Ears with covered, sound tips	Ears with exposed or injured tips
	Percent	*Percent*	*Percent*	*Percent*
Silks around middle portion of ear	27.1	54.2	17.3	18.2
Kernels near middle portion of ear	21.8	51.6	16.8	18.0
Pedicels near middle portion of ear	7.9	27.9	9.4	11.2
Vascular cylinder near middle of ear	3.2	12.3	3.0	5.6
Internal tissues at butt of cob	2.3	3.7	2.1	2.2

Three ears were found with infection in the butt of the cob only, 3 with infection in silk and butt of cob, indicating independent areas of infection, and 2 with infections in silk and kernels and butt of cob, also indicating independent infection at the butt. In 1 ear infections were found in butt of cob, vascular cylinder, and pedicels, and in another the same tissues plus kernels were involved. These were the only 2 in 1,240 plated ears where the fungus seems to have come into the kernels by way of the butt of the cob. In 378 ears, on the other hand, the fungus was clearly shown to have come in by way of the tip of the ear.

On September 2–5, about a month after pollination and when kernels were just ready to dent, only 7 percent of the ears well covered by husks were infected with *Fusarium moniliforme*. Kernel infection was even less prevalent, only 3.2 percent of the ears showing surface-borne kernel infection at this time. On succeeding dates, however, the percentage of infected ears increased progressively in considerable amounts (table 1). In ears that had been exposed to the weather by failure of shuck protection at the tip or that had been only slightly injured at the tip end by worms, infections averaged more than twice as many as in the first group mentioned (table 1). Infections with *F. moniliforme* were increased more than any other kind of infection by injuries at the tip of the ear or failure of the husk to protect it. In 1933, when only fully covered ears were used, observations were confined to sound ears and ears with worm-injured tips. In 1937, on the other hand, worm injuries were almost absent, but some of the ears used were incompletely covered by the husks. Both conditions were nearly equal in increasing infections with *F. moniliforme*.

Most of the kernel infection was on the surface of the pericarp. Colonies started at points where the kernel had been in contact with the silks (fig. 1, *B*). In a little later stage the entire pericarp surface carried the fungus (fig. 1, *C*). On September 2–5 none of the infections had penetrated the kernels sufficiently to escape being killed by surface sterilization. The same was true on September 13–15 and September 24–25 for well-covered and sound ears only (table 1). In ears exposed at the tips or slightly injured at the tip only by earworms, penetration by *Fusarium moniliforme* into the kernels at the middle of the ear came earlier and was more extensive. Boewe [2] found more than twice as much *F. moniliforme* rot in ears with open husks as in those with closed husks. The results herein reported, although the data are based on infection in apparently sound kernels instead of in rotted kernels, agree with Boewe's results.

CEPHALOSPORIUM ACREMONIUM INFECTION

Apparently the course of ear infection with *Cephalosporium acremonium* Corda may be by any one of three different routes. In fully half of the infected ears studied by method 1, the course began in the region of the silks. A colony on the surface of a kernel where the kernel had been in contact with the silk is shown in figure 2, *B*. In a large number of other ears, however, the first point of contact of the fungus with the kernel was on the shoulder where the kernel had been in contact with the glume. In some cases the glumes remained attached to the kernels and then the colonies appeared to arise from the glumes. A case in which the glume had become torn and the lower

[2] BOEWE, G. H. THE RELATION OF EAR ROT PREVALENCE IN ILLINOIS CORN FIELDS TO EAR COVERAGE BY HUSKS. Ill. Nat. Hist. Survey Biol. Notes No. 6. 19 pp. Urbana, Ill. 1936. [Mimeographed.]

end turned away from the kernel so that the origin of the colony is clearly shown is illustrated in figure 2, *C*. Under these circumstances, the fungus had, it seems, progressed down the ear by way of the glumes. As pedicels, vascular cylinder, and butt of cob in early cases of such infection were sterile, it is assumed that the fungus must have entered at the tip of the ear.

Less than 2 percent of the ears infected by *Cephalosporium acremonium* had been invaded by way of the butt of the cob. Various stages ranging from infections only in butt of cob to complete penetration through to the kernels were found. A case of incomplete penetration is illustrated in figure 2, *D*. In this case the fungus was found in butt of cob (not illustrated), vascular cylinder (center of dish), and pedicels. The latter, however, were infected only at the end that had been attached to the cob. The ends of the pedicels that had been attached to the kernels, and the kernels themselves, were sterile.

On September 2–5, 9 percent of the ears carried *Cephalosporium acremonium* infection. The fungus was confined to the silks or glumes and surface of the kernels. By September 13–15 prevalence of infection had increased considerably and cases of infection were found in all parts plated. At later dates, increases in infection were found only in the deeper parts, i. e., pedicels, vascular cylinders, and shanks. The prevalence of *C. acremonium* infection was influenced very little by whether the ears were well covered by the husks and were sound or whether they had been exposed at the tips or were slightly injured by earworms (table 3). This is surprising in view of the fact that the vast majority of the ears appeared to have been invaded by the fungus by way of the tip of the ear.

In other experiments (method 2) presented in table 2 there was stronger evidence that prevalence of *Cephalosporium acremonium* infection within the kernels was increased by injuries by worms or exposure at the tips of the ears.

GIBBERELLA ZEAE INFECTION

Only 9 ears of the 1,240 plated by method 1 showed infection with *Gibberella zeae* (Schw.) Petch. All these were found in 1935, and in every case the fungus had entered by way of the tip of the ear. Colonies on kernels originated somewhere on the crown half, indicating contact with silks. No cases of infection were found in pedicels, vascular cylinder, or butt of cob. It should be recalled that the ears were broken in the middle only, and that in all this work the ears used were sound in appearance except for occasional slight amounts of mold on the tip end. Thus the fungus was halfway down the ear before it caused visible rot. This was a greater distance than was usually found with *Diplodia zeae* in similar immature ears. Ears studied by method 2 showed a somewhat higher percentage of *G. zeae*-infected ears. Again, all the infection apparently originated by way of the tip end of the ear (table 2). Ears found with infections on the butt half were infected more extensively on the tip half. In other studies, however, the writer has seen a few ears with gibberella rot apparently starting at the butt.

NIGROSPORA INFECTION

Nigrospora oryzae (B. and Br.) Petch and *N. sphaerica* (Sacc.) Mason were both present in the material plated, but whether there

are valid reasons for separating this fungus into two species is still an open question. The term *Nigrospora* spp. is used here to cover both species. The earliest infections observed by method 1 were September 13–15, when 4 infected ears were found. One carried infection in the silks only, 1 on silks and kernels only, 1 at butt of cob only, and 1 in butt of cob and vascular cylinder only. Contrary to expectation, prevalence did not increase much with later platings. In 10 out of a total of 17 *Nigrospora*-infected ears found, infection appeared to have started at the butt of the ear; in 7 at the tip of the ear. Internal kernel infection occurred in only 2 of these 17 ears, and only in the October harvestings. As concluded from these figures and from the data in tables 2 and 4, *Nigrospora* may enter the ear at both the tip and butt ends, the latter being most common.

DIPLODIA ZEAE INFECTION

Infection with *Diplodia zeae* (Schw.) Lév. was very prevalent in 1933. Although only externally sound appearing ears were brought to the laboratory, when the husks were opened, 83 ears were found to be already visibly overrun by the diplodia fungus over a small to large part of the ear. These ears were not plated in the usual way by method 1 and are not included in the total count of 1,240 ears mentioned earlier. In addition, 28 sound-appearing ears, when plated in the manner described for method 1, also developed diplodia colonies. Seventeen of these ears were infected at the butt of cob only; 5 showed progressive stages beginning at the butt and involving vascular cylinder and sometimes pedicels and kernels. Six showed that the fungus had entered by way of the tip of the ear.

Pieces from the ears that were visibly partly overrun by the diplodia fungus were also plated but in a different manner. Sound-appearing kernels, pedicels, fragments from the vascular cylinder, and a piece from the center of the pith of the cob were removed and plated at a distance of 1 inch away from visible fungus development and also at a distance of 2 inches. After plating, 60 percent of the ears showed infection of the kernels at the 1-inch distance, 40 percent at the 2-inch distance. Infection showed most often in the kernels, least often in the pith. Thus in approximately 60 percent of the diplodia-infected ears, the invisible advance of the fungus was less than 2 inches ahead of the visible area of infection as viewed on the surface of the ear. These were early season infections. In later infections when ear development is more nearly mature, the situation is probably somewhat different, for in the latter case one frequently finds ears with the germs of all the kernels darkened by diplodia attack without evidence of this damage on the surface of the unshelled ears.

MONILIA INFECTION

A species of *Monilia* was a very common source of infection in corn ears in each year of the investigation. When the tissues were plated on potato-dextrose agar the colonies were a light-pink color, very much like *Fusarium moniliforme* or *Cephalosporium acremonium*, but more restricted in colony size and more delicate in texture. A colony is shown in figure 1, *B*, *f*. The spore sizes were 2.5 to 3 by 5 to 10 microns. In 1933 *Monilia* was not observed on the plates until September 25, but in 1935 in the first plating, September 4, it was the most prevalent fungus, being shown by 31 ears as compared with

25 showing *F. moniliforme*. This lead was maintained throughout the season. In 1937 *Monilia* was present to about the same extent as *F. moniliforme* in well-covered sound ears and was observed in the first tests of the season. Failure of husks to cover the ears did not aggravate infections by *Monilia*, and thus in exposed ears *F. moniliforme* was more prevalent. Because of the small size of the colonies, some of them were doubtless covered by other fungi and so a full record was not obtained.

The course of infection with this *Monilia* sp. was very much like that of *Fusarium moniliforme*, except that prevalence was not increased significantly by corn earworm injury or failure of complete husk protection. Entry into the ear was apparently exclusively in the region of the silks. The pedicels and vascular cylinder were readily invaded, and a small percentage of ears showed complete penetration through to the butt of the cob. Internal seed infection, however, was not found.

PENICILLIUM INFECTION

The plates were fairly free from penicillium when the ears were tested in September. In October when the husks became dry and somewhat loose, penicillium colonies were more abundant, especially in ears with incomplete husk covering or those with earworm damage at the tips. Almost all of this infection was confined to the silks and kernels. The prevalence of penicillium was highest in the October 16, 1935, plating, when the following percentages were recorded: Silks 37, kernels 33, pedicels, 1.5, vascular cylinder 1.5, butt of cob 3.0. A number of species were involved and no attempt at identification was made except that watch was kept for *Penicillium oxalicum*. This species causes a seedling blight of corn, and is identified comparatively easily. In 1933, of 28 ears found infected with penicillium, 18 were identified as *P. oxalicum*. But in 1935 and 1937 this species was not observed.

Extremely few cases of penicillium infection within the kernels were found in these experiments. In another experiment, however, in which mature ear corn, picked as seed ears, was dried slowly, considerable internal seed infection with penicillium was found (*5*).[3] According to the writer's observations in Illinois, the penicillia rarely penetrate into normal uninjured corn kernels as long as they are in an actively growing condition, and if the ears are dried rapidly as soon as they are mature very little internal infection is found.

CORN EARWORM INJURY AND FUNGUS INFECTION

Some aspects of the relation of slight earworm injury and incomplete husk protection at the tip of the ears to fungus infection at the middle of the ears have already been discussed under the different fungus name headings, and some of the data have been given in table 1. Another experiment dealing specifically with earworm damage was conducted as explained under method 2, and the results are given in table 2. It should be emphasized that these experiments were confined to ears with worm injuries only at the tip. They were not concerned with the identity of fungi at the injured location, but rather

[3] Italic numbers in parentheses refer to Literature Cited, p. 441.

with internal infection in kernels at some distance away from the injury.

It is already well known that corn earworm damage is followed by increased corn ear rot by *Fusarium moniliforme*. In the present work, based on kernel infection rather than rot, *F. moniliforme* infections were similarly increased most by earworm damage. Next in order were infections by *Gibberella zeae*, *Nigrospora* spp., and *Cephalosporium acremonium*. Infection with the last two was only slightly influenced by earworm injury. Infection with *Diplodia zeae* was apparently not at all affected by this factor.

Ears with worm injuries that had become moldy (figure 3, *C*) had higher percentages of kernel infection, on the average, than worm-injured ears that were free from mold. In general, it appears safe to assume that the injuries that became moldy occurred earlier, when the ears were more immature and more susceptible to infection, than injuries that did not become moldy.

INFECTION AT BUTT OF COB IN RELATION TO COB DISCOLORATION

As the season advances, fungi can be isolated in progressively greater numbers from the internal tissues where the shank breaks off from the cob and discolorations of these tissues become more pronounced. This situation was especially evident in 1933. On October 23 and 24, 546 apparently sound ears were harvested with the shanks intact, internal tissues from the butt of the cob were plated as explained under method 1, and the ears were classified into 4 groups according to the appearance of the butt of the cob (fig. 4). All ears were well covered by the husks and only those that appeared entirely sound after the husks had been removed were used (fig. 3, *A*).

The term "slightly discolored," applied to butts of cobs, refers to light-yellow, drab, tan, or pink colors, while "badly discolored" refers to strong drab, tan, brown, pink, or red colors. Nearly all of the cobs had a natural pink, red, or brown color of the internal vascular cylinder, even those classified as "white" at the butt. Usually this natural color did not extend down to the place where the shank broke off, but occasionally no doubt it did so. When this happened it was difficult to distinguish between natural color and discoloration caused by infection. In corn genetically pure for white internal cob color, much better separations could be made.

In spite of the fact that some ears probably carried natural genetic colors which were classed as "discolored," the relationship between discolorations and the presence of fungus infection in the interior tissues at the butt was outstanding (table 4). The correlation between extent of butt of cob discolorations and infections by *Diplodia zeae*, *Nigrospora* spp. and *Fusarium moniliforme* was especially good (table 4). Infections with *Cephalosporium acremonium*, *Gibberella zeae*, and *Penicillium* spp. bore little or no relationship to shank discoloration. *Nigrospora* infection was strongly related to shredded shanks.

More ears were infected with *Diplodia zeae* and *Nigrospora* spp. at the butt of the cob than in the kernels. This fact, with other evidence discussed earlier in this paper, leaves no doubt that most of the ear infections with these organisms came in through the butt of the cob.

There were, however, exceptions with *Nigrospora*, and many exceptions with *Diplodia*. With *Fusarium moniliforme*, on the other hand,

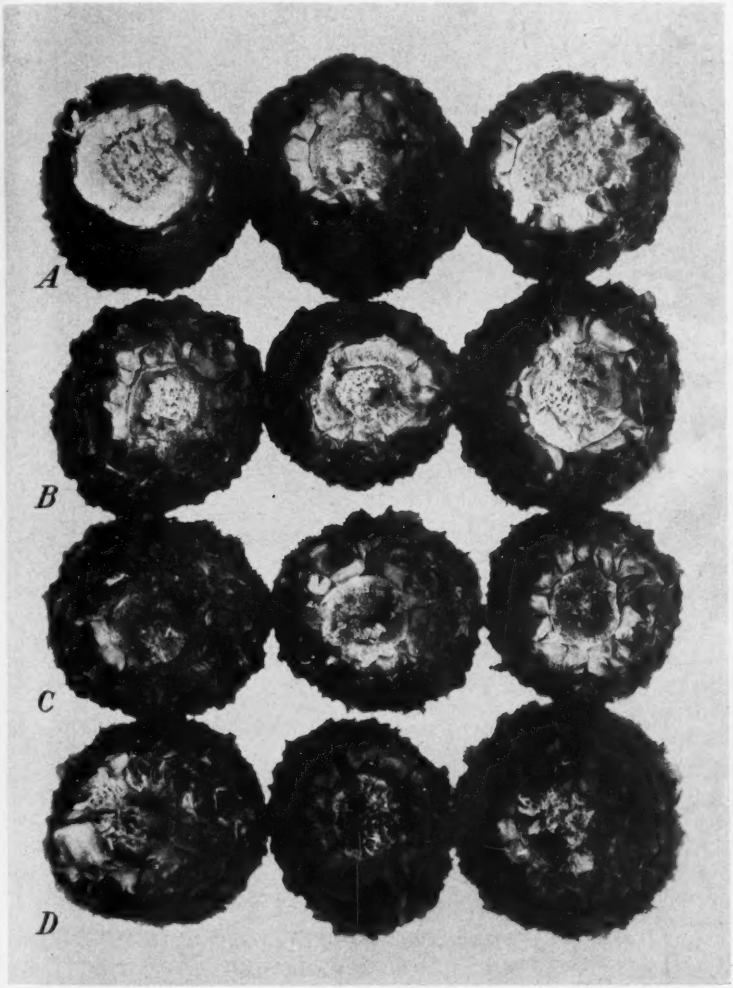

FIGURE 4.—Butts of cobs: *A*, Bright, sound, healthy appearance; *B*, slightly discolored; *C*, badly discolored; *D*, discolored and pith disorganized so that the vascular strands appear shredded. These butts are typical of the classes in table 4.

kernel infection was more prevalent than butt of cob infection. This corroborates the results obtained by method 1, namely, that kernel infections with *F. moniliforme* nearly always precede butt of cob infections.

TABLE 4.—*Ears of Reid Yellow Dent corn showing fungus infection when tested a butt of cob and in kernels around central part of ear after having been classified according to extent of discoloration at the butt of cob, Urbana, Ill., 1933*

Kind of ears and fungus isolated	Ears infected when cob at butt was—			
	White (141 ears)	Slightly discolored (185 ears)	Badly discolored (130 ears)	Discolored and shredded (90 ears)
Ears with butt of cob infection:	*Percent*	*Percent*	*Percent*	*Percent*
None	79.4	60.0	34.6	15.6
Diplodia zeae	2.8	14.1	28.5	34.4
Nigrospora spp.	2.8	7.6	6.1	23.3
Fusarium moniliforme	5.0	7.6	17.7	17.8
Cephalosporium acremonium	.0	.5	.0	.0
Gibberella zeae	.0	1.6	.0	.0
Penicillium spp.	5.7	4.3	8.5	6.7
Miscellaneous and unidentified	4.3	4.3	4.6	2.2
Ears with kernel infection:[1]				
None	65.2	55.1	42.3	25.5
Diplodia zeae	4.3	11.9	24.6	27.8
Nigrospora spp.	.0	.0	.0	11.1
Fusarium moniliforme	27.7	31.4	35.4	38.9
Cephalosporium acremonium	2.1	2.7	3.1	2.2
Gibberella zeae	.7	.0	.8	.0
Penicillium spp.	.7	1.6	.0	1.1
Miscellaneous and unidentified	1.4	4.3	3.1	4.4

[1] Recorded as an infected ear when 1 or more of the 8 kernels tested per ear by surface-sterilizing and plating showed infection. Some ears showed the presence of more than 1 kind of fungus, and each was recorded.

POSITION OF INTERNAL SEED INFECTION

The path of infection to the kernel having been followed, it is desirable to know where the fungi enter the kernel and which tissues are most readily invaded. Manns and Adams (*7*) found that in mature, sound-appearing kernels the mycelium of *Fusarium* and *Cephalosporium* often occurred in the tip cap (fig. 5, *a*) and especially in the cavity between the tip cap and the brown to black layer covering the scutellum (fig. 5, *e*). They state: "The development of the fungus from the cap into the germ and endosperm appears to be restricted by the black layer." In some cases, however, they found fungus mycelium also in the cavity around the radicle and plumule.

Johann (*4*) called the dark layer, mentioned above, the "closing layer," and found that it was rather impervious to fungus penetration once it had formed, but in the earlier stages of kernel development this protection was lacking.

Branstetter (*1*) cut surface-sterilized, sound-appearing kernels into five pieces. When infection occurred it was always present in the section nearest the tip end, and in 31 percent of the kernels infection occurred only in this area.

An experiment was set up to determine in which particular tissues of the kernels infection occurs. The technique is described under method 3. Ears from the 1938 crop on the station farm were used.

Infection was found to be most common in the tip cap. This agrees with the results of other investigators. Next in decreasing order of infection prevalence were tissues of the germ, floury endosperm, and horny endosperm (fig. 5). This was true of all the fungi studied (table 5). When infected ears showing no visible symptoms on the

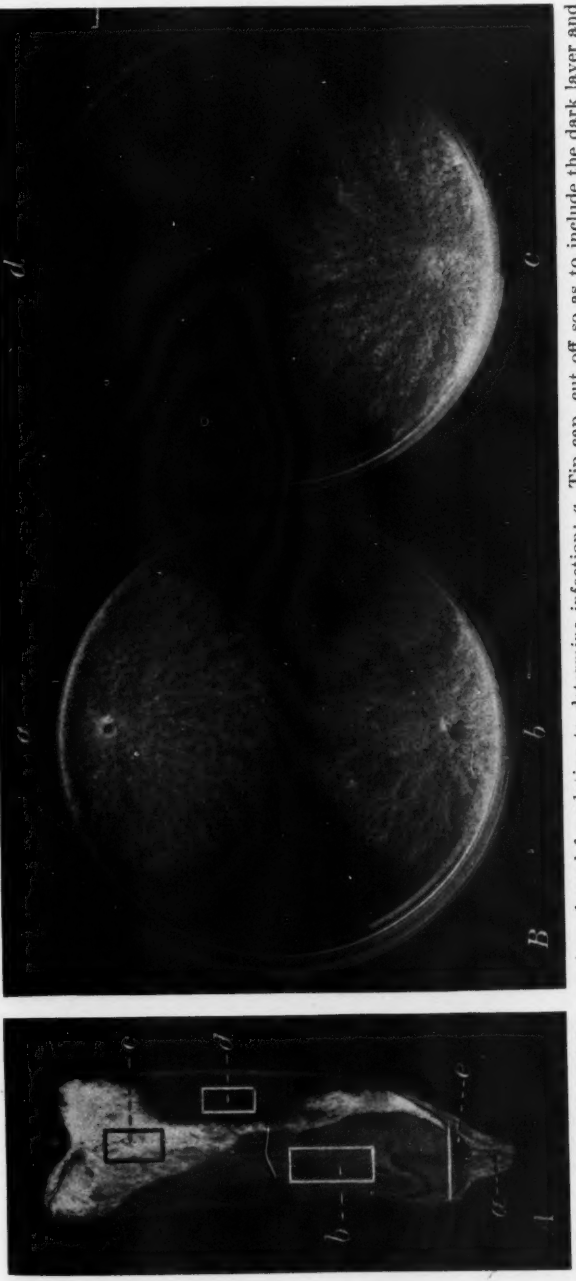

FIGURE 5.—*A*, Parts of the corn kernel used for plating to determine infection: *a*, Tip cap, cut off so as to include the dark layer and hollow space at *e*; *b*, central part of germ; *c*, floury endosperm; *d*, horny endosperm. *B*, The letters correspond to the same letters in *A*, showing, in this case, *Diplodia zeae* colonies arising from all the tissues plated except the horny endosperm.

TABLE 5.—*Fungus infection in different parts of well-developed mature Reid Yellow Dent corn kernels studied by method 3; fragments dissected out under aseptic conditions and plated on agar medium: Class 1, kernels showing no outward signs of infection; class 2, kernels slightly discolored by infection; Urbana, Ill., 1938*

Class	Cause of infection	Ears used	Kernels plated	Infection in tip cap	Infection in germ	Infection in floury endosperm	Infection in horny endosperm
		Number	*Number*	*Percent*	*Percent*	*Percent*	*Percent*
1	*Diplodia zeae*	6	30	96.7	43.3	20.0	6.7
	Fusarium moniliforme	11	55	76.4	21.8	1.8	.0
	Nigrospora spp	5	25	48.0	8.0	4.0	.0
	Cephalosporium acremonium.	4	20	80.0	.0	.0	.0
2	*Diplodia zeae*	19	95	100.0	98.9	82.1	28.4
	Fusarium moniliforme	33	165	99.4	83.0	70.3	48.5
	Nigrospora spp	10	50	100.0	24.0	12.0	4.0
	Cephalosporium acremonium.	2	10	100.0	40.0	30.0	.0
	Penicillium spp	4	20	100.0	95.0	60.0	35.0

kernel were selected, not all kernels on the ear carried infection, but when infected ears having kernels with slight discolorations were selected, infection was practically 100 percent (classes 1 and 2 respectively, table 5). *Cephalosporium acremonium* in class 1 showed least ability to spread into other parts of the kernel from the tip cap. In class 2, both *C. acremonium* and *Nigrospora* spp. made the least extensive growth into various parts of the corn kernels. Discolorations from *C. acremonium* consisted of white streaks (fig. 6, *B*). In the writer's material, this fungus did not cause significant kernel rot or discolorations beyond the streaking just mentioned. With regard to *Nigrospora*, it should be borne in mind that the kernels used were well developed and had a well-differentiated horny endosperm. Thus these results do not apply to infections that arrest the development of kernels so that the horny endosperm is poorly developed. In the latter case the germ becomes badly disintegrated (*2*).

Another tissue plated from these kernels was the pericarp from a considerable area of the middle of the back of each kernel. After soaking overnight, the pericarp can be pulled away easily, the break occurring near the testa (*4*). By the method used, the pericarp usually plated sterile. It was discovered later that the sterile pericarp platings were due not always to absence of infection but to penetration of the disinfectant used for surface sterilization.

NATURE OF WHITE STREAKS ON PERICARP

A special study was made of the nature of the white streaks often found on the pericarp of infected kernels (fig. 6). That these streaks may take form rather late in the development of the kernel was shown in 1938 when corn was lodged artificially on September 16, about 15 days after denting had been completed and when the moisture content of the kernels was approximately 35 percent. When harvested, kernels with streaked seed coats were numerous in the lodged corn, but rare to absent in the standing corn. This type of streaking occurring in the field may be of a different nature from that developing when corn is being germinated in a germinator. The latter has been mentioned by Manns and Adams (*7, p. 504*), who state: "As a rule, the fungus ramifies between the pericarp and epidermis, resulting in pronounced discoloration of the pericarp and involving the lower half

of the kernel. Often the discoloration will appear as irregular streaks, extending from the germ end toward the crown."

Streaked kernels developing in the field invariably showed infection when surface-sterilized whole kernels or tip ends of the kernels were plated. Fungi isolated were *Fusarium moniliforme*, *Cephalosporium acremonium*, and *Nigrospora* spp. Streaks extending up to the top of tbe crown were most often associated with *F. moniliforme*. *Nigrospora* usually caused wider streaks than the others and these had a tendency to coalesce near the tips of the kernels. Streaks caused by *C. acremonium* most often were limited to the lower half of the kernels, and had a tendency to coalesce at the tips. Figure 6, however, also shows some exceptions to these statements. At times the streaks

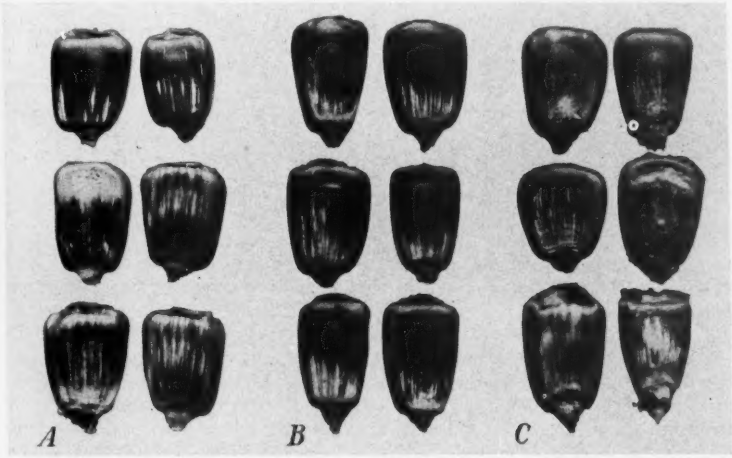

FIGURE 6.—Kernels with white streaks on the pericarp caused by infection with: *A*, *Fusarium moniliforme*; *B*, *Cephalosporium acremonium*; *C*, *Nigrospora* spp. Identified by surface-sterilizing and plating.

were distinctive enough to permit one to identify the infection with a fair degree of accuracy. Durrell (*2*, *fig. 4*) illustrated streaks caused by *Basisporium gallarum* (*Nigrospora* sp.), and Edwards (*3*, *fig. 20*) showed streaks caused by *Gibberella fujikuroi* var. *subglutinans* (=*F. moniliforme* var. *subglutinans*).

By examination with a high-power binocular microscope, fissures in the pericarp surface coinciding with the white streaks can be seen, although this surface appears smooth and intact to the naked eye. Free-hand sections were made of mature kernels with each of the three kinds of infection mentioned above. Pieces of kernels, after being disinfected, were soaked overnight in water, cut, and stained with Pianese III B. This not only demonstrated broken-down cells in the pericarp but also gave good differentiation of fungus hyphae in red as contrasted to the pericarp cells in green. Pieces of pericarp cleared in chloral hydrate showed the cells to be very pitted and very long. This latter fact seems to be the reason for the linear direction of the streaks. Additional sections were cut also from pieces taken from the back of streaked *Fusarium moniliforme*-infected kernels

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which had been killed in Gibson's fixative, embedded in paraffin, and stained in gentian violet and Pianese.

The streaks were definitely not caused by the mycelium growing between the pericarp and testa and thus lifting the colorless pericarp away from the yellow layers beneath. What actually happened was that the fungus caused a break-down of the pericarp cells, often at the surface (pl. 1, *B*, *C*) but sometimes in the middle area (*F*) and sometimes next to the testa (*D*). The normal cells are very thick-walled and possess considerable transparency. After corrosion by infection, only shriveled membranes remain and these areas have lost their transparency and have taken on a chalky appearance.

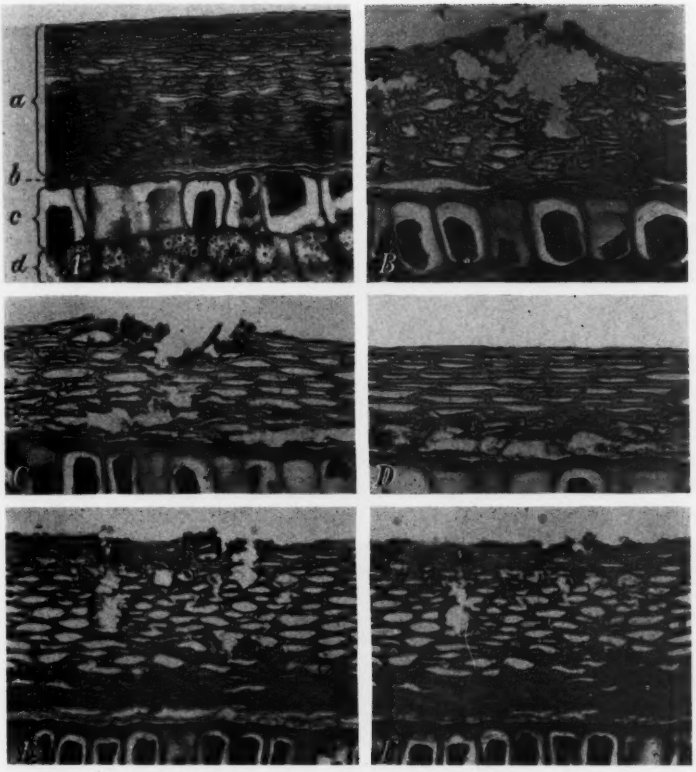
Infection of the pericarp undoubtedly takes place before complete maturity and appears to be effected in two ways: (1) Internally at the junction with the tip cap, and (2) externally by action of the fungus on the surface. Once a cell has been entered, progression is linear because the cells are very long. Progression at right angles takes place slowly. The mycelium can clearly be seen in the lumen of the cells and in the pits. However, passage from cell to cell through the pits does not seem to occur readily; spread sidewise is accompanied by considerable disintegration of the cell walls.

DISCUSSION

In corn ears that were well protected by the husks and had not been entered by earworms, a surprisingly large number were found to be sterile until practically mature. As a 3-year average for the dates September 2–5, 13–15, 24–25, October 5–7, and 15–18 the corresponding percentages of sterile ears were 77.6, 68.2, 45.3, 46.4, and 48.0. It would appear as though a stationary point were reached about September 24–25, but this is not true with respect to the whole population because the percentage of ears well covered by husks and free from earworm attack is steadily decreasing during this period. Perhaps the plugging of the channel through the husks at the end of the ear by the silks is a help, and it is highly probable that, prior to complete maturity, the tissues involved along the path of infection are a poor medium for the growth of saprophytic fungi.

Nearly all fungi in corn ears were found to enter either by way of the channel through the husks at the tip of the ear or at the base of some of the husks and thence through the shank. One way in which infection may reach the shank after traversing the length of the husk has previously been illustrated (*6*, fig. 27). A local shank infection which resulted in entry into the ear is shown in figure 7, *A*. Sometimes, however, the ear becomes infected first and the shank second. The appearance of a rot at the butt end of the ear does not necessarily mean that the infection entered at the butt. In experiments with artificial inoculations (unpublished) it was observed that, after the ears had dented, some of the inoculations made with *Diplodia zeae* at the tip of the ear resulted in infections like that in figure 7, *B*. The fungus apparently made its way down the ear without prominent visible signs, and produced rot at the lower end where moisture was highest.

Occasionally *Diplodia* may progress from an infection in the stalk through the shank and cause ear rot. This was proved by making stalk inoculations in the internode beneath the node that bears the



A, Section through the pericarp of a sound noninfected kernel of corn from the side opposite to the germ and cut at right angles to the long axis of the kernel: *a*, pericarp; *b*, testa; *c*, aleurone; *d*, horny endosperm.

B–F, Sections through white streaks of *Fusarium moniliforme*-infected kernels similar to those shown in figure 6: *B*, Tissues severely broken down in the outer half of the pericarp; *C*, disorganization, starting at the surface, has progressed through the entire thickness of the pericarp; *D*, serial section from the same kernel as in *C* but at some distance from it, showing disorganization only near the testa; *E*, disorganization of cells resulting in long internal channels; *F*, serial section from the same kernel as in *E*. Some of the sections, especially *E* and *F*, show the dry dormant mycelium. × 265.

shank. A small but significant increase in diplodia ear rot was obtained in this way.

It was supposed at one time that ear infection with *Cephalosporium acremonium* was the result of systemic infection of the plant (*8*).

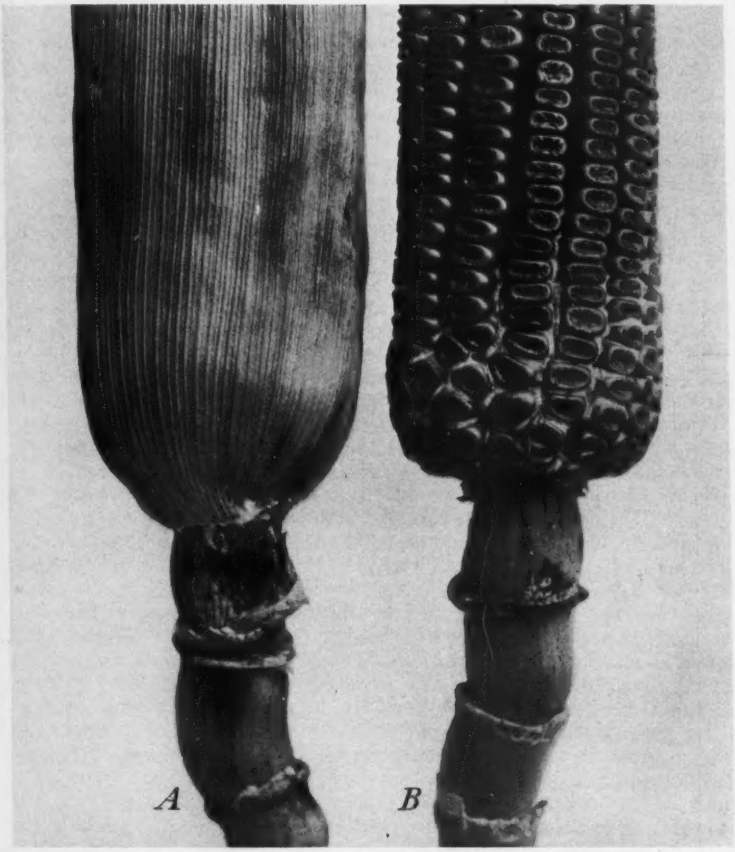


FIGURE 7.—Corn ears rotted at the butt end by *Diplodia zeae: a*, Infection started at the base of husks; *b*, infection started at the tip of the ear. This ear was artificially inoculated at the tip end, but rot developed primarily at the butt where moisture was highest.

In the experimental work herein reported, this was not true in a large majority of the cases. There were, however, some clear-cut cases (fig. 2, *D*) of *C. acremonium* infection that came via the butt of the ear. Whether or not these cases were the result of systemic infection was not determined.

Having made contact with the ear at the tip end, some fungi ramify pretty much to all parts of the ear before kernel penetration takes place, while in very immature ears some others may cause penetration at a distance of only a few inches behind the front ad-

vance of the mycelium. Environmental conditions, variety of corn, and stage of development of the ears are important factors determining ability of fungi to overrun ears and penetrate into kernels. In general, if one were to classify the fungi entering at the tip of the ears according to their relative ability to penetrate into kernels so that the fungus persists after surface sterilization, the ranking, according to information at hand, in descending order would be: *Diplodia zeae*, *Gibberella zeae*, *Nigrospora* spp., *Fusarium moniliforme*, *Cephalosporium acremonium*, and *Pencillium* spp. *Nigrospora* spp. occupy an uncertain place in this ranking because, in general, they become parasitic only under certain host responses to the environment. If the ranking were according to ability to cause rot, *C. acremonium* would come last. This ranking, of course, is irrespective of prevalence.

Entry into very young kernels may apparently be effected anywhere through the pericarp and testa by *Diplodia zeae* and *Gibberella zeae*, but as the kernels develop, the testa offers increasing resistance (*4*) and finally even these fungi are limited to entry via the tip cap. If any of the other fungi mentioned in this paper ever enter into the internal tissues enclosed by the testa of undamaged corn kernels in any way except via the tip cap, the occurrence seems to be rare. However, when mechanical damage or growth cracks have occurred while the kernels are still juicy, *Fusarium moniliforme*, *Pencillium* spp., and other fungi have been found to enter at the injured place.

When entry is via the tip cap, after denting has occurred, the fungus may pass up into the pericarp and also into the internal tissues, but apparently it cannot pass through the testa from one to the other. Pericarp infection can usually be diagnosed by the presence of white, pink, or dark streaks. It is found most often when infected ears dry or mature very slowly as, for instance, in lodged corn when the outer husks are in contact with the ground or in standing corn when protracted wet weather occurs during the final maturing period. Infection in the pericarp is easily killed by ordinary surface disinfection, but infection in the tip cap and internal parts persists. After entering into the interior of the kernel, *Diplodia* ramifies most easily through the aleurone, according to Johann (*4*). In the present work, *Diplodia zeae*, *Fusarium moniliforme*, *Cephalosporium acremonium*, and *Nigrospora* spp. were all found more often in the germ than in the soft or horny endosperm. This agrees also with the appearance of infected kernels when cut open; i. e., the germ is the area that is most often discolored.

SUMMARY

The method of experimentation consisted of plating on agar medium natural tissues, unsterilized, from selected parts of corn ears in various stages of development; the plating of mature surface-sterilized whole and dissected corn kernels; and the preparation of histological sections of corn kernels.

Fusarium moniliforme was the most prevalent fungus in corn ears. In nearly all cases the fungus entered in the region of the silks, the kernels became contaminated at points of contact with the silks; thence infection spread to the pedicels, vascular cylinder, and finally the shank. In very few cases did infection proceed in the opposite manner, from shank to kernel. Internal kernel infection in sound-

appearing kernels did not become established until the ears were approaching maturity.

A large percentage of infection with *Cephalosporium acremonium* also took place in the manner just mentioned. Another common method of infection was of a type in which the fungus made contact with the lower half of the kernel surface. Apparently the fungus progressed down the ear in the area in which the glumes are located. A smaller percentage reached the kernels by way of the butt of cob, vascular cylinder, and pedicels. Internal kernel infection became established when the ears were approaching maturity.

Gibberella zeae infection practically all started at the tip ends of the ears and progressed down the ear most rapidly in the region of the silks.

Diplodia zeae and *Nigrospora* spp. caused infection by entering at both the tip and butt ends of ears with a slight majority in favor of the latter. Fungus penetration at the butt was very largely the result of local infections on the shank. *D. zeae* caused a more active and more generalized rot in the wake of the advancing mycelium than any of the other fungi.

A species of *Monilia* was very prevalent in some seasons. It entered in the region of the silks and progressed over the surfaces of the kernels and invaded the pedicels and vascular cylinder. Internal kernel infection, however, failed to take place.

Penicillium infections behaved very much like *Fusarium moniliforme* infections except that first appearances were later in the season, prevalence was lower, and internal kernel penetration into sound-appearing kernels was practically absent.

Exposure of the tip of the ear from incomplete husk protection or slight injury at the tip from earworms caused marked increases in infection by some fungi.

Discoloration at the butt of the cob where the shank was broken off was found to be a strong indication of infection at the butt and also of the kernels of ears that had previously been selected for good husk covering and freedom from worm damage.

White streaks on the pericarp of kernels were found to be caused by the invasion of the pericarp by *Fusarium moniliforme*, *Cephalosporium acremonium*, or *Nigrospora* spp. The white color was due to the disintegration of cells which caused them to lose their transparency and take on a chalky appearance.

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STUDIES ON THE VIROPLASM HYPOTHESIS[1]

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INTRODUCTION

Fifteen years ago it was suggested from this laboratory that some part of the normal protoplasm or cytoplasm of one species, if properly introduced into the cells of another species, might find conditions compatible for growth in that species, and bring about the abnormality which is known as virus disease (*6*).[2] This "viroplasm" hypothesis[3] was based largely on results secured with extracts from apparently healthy potato plants inoculated into other members of the Solanaceae. Subsequent evidence has shown that practically all standard varieties of American potatoes are symptomless carriers of the mottle virus or ring spot virus in question, and that the hypothesis was untenable. Other evidence would be required to support the viroplasm hypothesis if it were to remain even a probable theory. Not many investigators have given this theory of the origin of viruses serious second consideration, but its possibilities have appealed to some pathologists who have encouraged further efforts to determine its validity.

It must be assumed that the possible compatible combinations under the viroplasm hypothesis are relatively rare, otherwise a legion of different viruses in epidemic proportions would be expected. If such combinations are as rare for example as chimeras, compatible interspecies grafts, or natural crosses between species, the number of viruses actually originating spontaneously in nature through transmission of foreign protoplasm by certain insect species or other natural means might be expected to be roughly proportional to the number of natural viruses known to exist. The possible number of compatible combinations of the "viroplasm" type which may be attempted is, of course, enormous, even if it were limited only to related species, e. g., within a family. The introduction of still other circumstances, such as the possible variations in artificial methods of transfer of protoplasm or the probability of the labile forms of protoplasm (attenuated forms) requiring repeated culturing or transfer in new hosts in order to secure the full expression of symptoms are of such a nature as to discourage further experimental efforts in this direction.

Even when limiting the trials to only family, as was done in the investigation to be reported, the result secured soon become so intricate and involved that it is necessary to repeatedly eliminate interesting possibilities from further trial. That unusual compatibility of a similar nature may exist between widely different plant families as well is suggested by Daniel's (*2*) combinations in grafts of such plants as sunflower and melon, cabbage and tomato, maple and

[1] Received for publication May 8, 1941.

[2] Italic numbers in parentheses refer to Literature Cited, p. 453.

[3] The use of the term "viroplasm" in this connection was first suggested to the writer by Dr. H. H. Whetzel of Cornell University.

Journal of Agricultural Research,
Washington, D. C.

Vol. 64, No. 8
April 15, 1942
Key No. Wis.-132

lilac. If such unions are to be taken as any indication of the possibilities of the existence of viroplasm, the chances of securing a compatible combination by trial and error are extremely small.

The experiments presented in the present paper yielded no positive proof in support of the validity of the viroplasm theory. The efforts are significant rather as demonstrating the problems to be expected in further studies of this type. Nevertheless, the results obtained are of some interest and value since they have brought to light two hitherto apparently unknown viruses, as well as what appears to be an allergic response in plants to the metabolic products of another plant species.

An abstract of this paper was published earlier (7).

EXPERIMENTAL METHODS

The Leguminoseae were chosen originally for the purposes of this study chiefly because many genera and species were obtainable from collectors of this plant family [4] and because this group is known to be affected by a considerable number of viruses. Many species of this family are not suitable for test plants either because of their relatively slow growth or because of leaf size or shape. However, the number of possibilities of cross inoculation are still great, even though only a few species are chosen to be inoculated with extracts from the others. The common bean (*Phaseolus vulgaris* L.) is an ideal test plant and consequently most of the inoculations were made to varieties of this species. The variety Stringless Green Refugee was always used unless otherwise stated. Fresh extract from apparently healthy species of legumes was transferred by the wiping method (now often used in virus studies) to the primary leaves of young healthy bean plants. Carborundum powder was regularly used with the inoculum in order to increase the probabilities of transfer, although its use was later shown to be often unnecessary.

The use of inoculum from young plants produced from seed has a distinct advantage over the use of inoculum from vegetatively propagated plants like the potato, since it is not at all likely that all individual plants are symptomless carriers of a virus as in the case of the potato (6). Occasional seed transmission of known viruses in legumes is not unlikely, and special precautions should of course be taken to avoid confusion through such a possibility. Beans used as test plants were obtained from "mosaic-free stocks" and rogued further for any signs of the disease before being used for test plants. It may be stated here that no instance of the seed-transmission of a previously described virus was noted in the trials except that of ordinary bean mosaic virus.

The plants were grown, and all inoculations made, in a greenhouse at a temperature averaging about 80° F. The plants to be inoculated were grown in 4-inch pots, with five beans planted in each pot. All small or abnormal seedlings were weeded out, usually leaving only three or four plants to the pot. Two pots of test plants (seven or eight plants) were commonly inoculated with each extract tested. Records were taken on all inoculated plants from 5 to 10 times at intervals of 1 to 3 days, always comparing them with uninoculated plants of the same age or other forms of control plants.

[4] The writer is indebted to the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and to many individuals for furnishing seeds of the species used.

The physical properties of the viruses isolated were tested in the same manner as previously described from this laboratory.

EXPERIMENTAL RESULTS

One hundred and twenty-two species of legumes representing 50 genera were grown and inoculated to the bean and certain other test plants. It seems unnecessary to list these genera or species in the present connection, or to present in detail the large number of negative results secured from the inoculations. With some species only a single inoculation trial was made, whereas with others up to 20 and, in one instance, over 100 separate series of inoculations were made from a single species to over 700 test plants. It is logical to assume that some known or unknown viruses might be carried through the seed of certain species and that the species involved might act as a symptomless carrier. One hundred percent seed transmission of a previously existing virus would not be expected, although 100 percent of compatibility might be expected or assumed to be necessary for proof on the basis of the viroplasm hypothesis. Repeated trials, in which single healthy plants were used as a source of inoculum, became especially desirable where any viruslike symptoms had been expressed in any prior trials between two species. If apparently healthy individual plants of a single species continued to yield responses on the inoculated host, the results naturally became most suggestive of those to be expected or assumed on the basis of the viroplasm hypothesis. Such an ideal result is obviously not necessary to the hypothesis. Quite as likely, positive results may be expected only occasionally, just as is the case of grafting or hybridization between species with a low degree of compatibility.

AN ALLERGIC REACTION IN PLANTS

When extracted juice of the Tangier pea (*Lathyrus tingitanus* L.) is wiped on the young primary leaves of the bean (*Phaseolus vulgaris* L.), the developing new secondary leaves become mildly chlorotic, vein-cleared, and somewhat stunted in growth as compared to control plants (figs. 1, 2). Although the symptoms may be modified to some degree by environment, they are retained throughout the life of the plant. The symptoms are so similar to those which may be expected in a relatively mild virus disease that experiments were continued with this material for several weeks in the belief that a virus was concerned. On this assumption, separate inoculations were made to the bean with over 100 individual Tangier pea plants (fig. 3) grown from seed secured from three sources. Typical symptoms were always obtained, suggesting either that all Tangier peas were symptomless carriers of a virus transmitted 100 percent through the seed or that the results supported the viroplasm theory. During the course of these trials, efforts were also directed toward serial transmission of the reaction and studies on properties of the agent, with what at first appeared to be unreliable results as a consequence of uncertain symptom expression. In some, but not in all instances, mild symptoms were secured on the first transfer from the original inoculated bean to bean, but not on the second transfer from the bean.

It was not until the results of certain property studies were completed that it became clear that a virus was not concerned with the

symptoms in question, e. g., the Tangier pea extract was found to withstand autoclaving at 115° C. for 30 minutes or longer without any reduction of its ability to reproduce the typical effect on the bean plant. The substance was not harmed by long aging in extract,

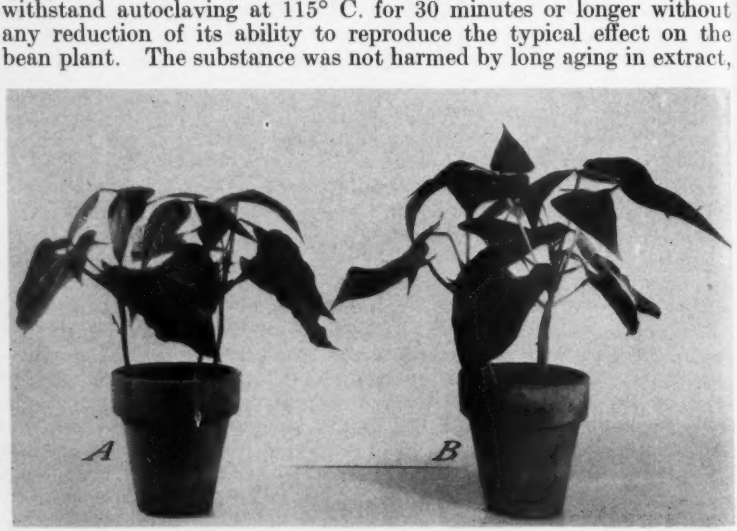

FIGURE 1.—*A*, Allergic type of reaction on bean plants inoculated with extract from healthy Tangier peas, showing typical mild stunting and chlorosis on first trifoliate leaves. Note absence of injury to inoculated primary leaves. *B*, Uninoculated control plants.

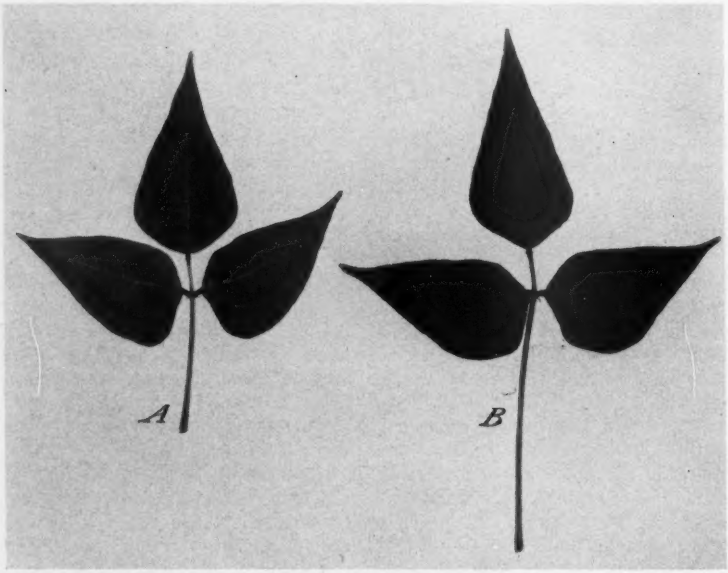

FIGURE 2.—*A*, Trifoliate leaf of bean showing mild stunting, general chlorosis, and some vein-clearing in response to the inoculating of the primary leaves with extract of the Tangier pea. *B*, Leaf from uninoculated control plant.

by freezing, or by drying for several months. The extract, however, rapidly lost its activity on dilution with water beyond 1 to 25. The active substance is filterable and capable of dialyzing through osmotic membranes. It is entirely extractable with water from the tissues

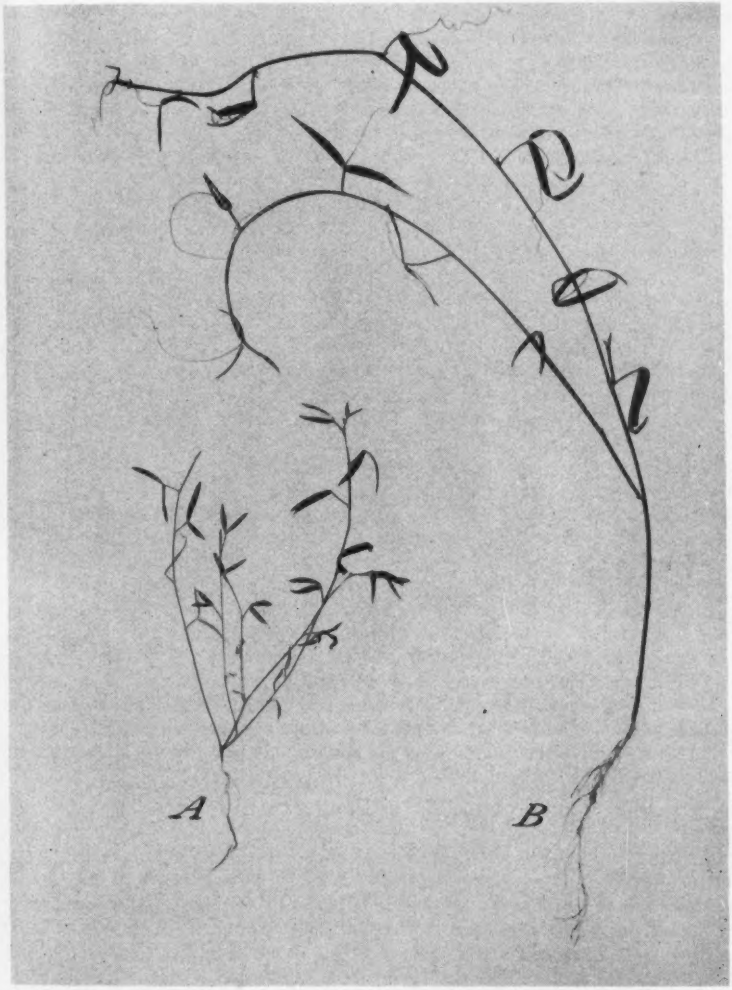

FIGURE 3.—*A, Lathyrus pusillus; B, L. tingitanus* (Tangier pea). Inoculation to bean plants with extract from the former species yielded the bean leaf wilt virus, and inoculation with the latter produced an allergic reaction in the plants.

of the Tangier pea, but nothing is known of its solubility in other substances or its reaction to chemicals, except that it appears to be denatured by cow's milk. The product does not appear to be present

in the seed, but it soon develops in the germinating seed. Symptoms similar to those produced on the Refugee bean were secured on other bean varieties, although only doubtful signs were observed on the varieties Robust and Great Northern. Velvetbeans, soybeans, and cowpeas were affected, but some other legume species, including *Phaseolus lathyroides* L., were not. *Lathyrus pusillus* L. appears to contain the same agent as the Tangier pea, but in lesser amounts; it does not appear to be present in *Lathyrus hirsutus* L.

The behavior of the extract from the Tangier pea suggests that it is a toxic substance, but it should be explained that no appreciable effect or symptom is observable on the primary leaves to which it is applied, the symptoms developing only on secondary leaves formed later. The symptoms on the bean are, therefore, systemic rather than local in character. The active substance is not toxic to viruses, bacteria, or fungi, according to preliminary trials. Since Tangier pea hay is occasionally grown for fodder, it is presumably not toxic to animals.

Although no further investigation of the Tangier pea extract was made, the writer is inclined to interpret the results as an allergic response, despite the fact that such a reaction has apparently not been previously described on plants. Hassis (*4*) has also noted chlorotic symptoms on tulip plants inoculated with healthy narcissus juice, even when heated to 100° C. for 20 minutes before inoculation. This symptom is therefore believed to be due to some cause other than a virus, although no further interpretation of the nature of the response is suggested. According to Rowe (*9*), Doerr's generally accepted definition of allergy includes "all abnormal and specific reactions of a body to foreign ordinarily innocuous substances." The action of the Tangier pea extract on the bean plant, everything considered, approaches such a reaction more nearly than that of any other biological response with which the writer is acquainted.

Although the experiments with the Tangier pea were disappointing and delayed progress on the original problem, they nevertheless encouraged further investigations on the viroplasm theory. This allergic type of behavior, peculiarly, appears to be an intermediary stage between a demonstrable and a hypothetical phenomenon, since it shows that a metabolic substance from one plant can be systemically introduced into another plant and thereby set up a series of reactions which suggest some small degree of transmissibility.

THE BEAN LEAF WILT VIRUS

During the course of the inoculations to the bean with extract from other apparently healthy legume species, two viruses were found which appear to be new and distinct from other known legume viruses. Two additional viruses were secured which appeared to be mild strains of ordinary bean mosaic; consequently their origin in the experiments was more uncertain, and they were not studied in further detail. It is logical to believe that all these viruses preexisted, and that they were transmitted through the seed of the host plants, which were also acting as symptomless carriers. No other claim is made at this time in the absence of further positive proof, but the viruses themselves are of interest and will be briefly described.

One of the viruses, to which the writer has applied the common name "bean leaf wilt virus," was obtained from *Lathyrus pusillus*

(fig. 3). This species showed no signs of disease at any time, but the plants were small for favorable observation. The virus was secured from only about 25 percent of the plants tested. The symptoms on Refugee bean are largely limited to the primary inoculated leaves. which usually wilt and dry up without developing preliminary necrotic lesions (fig. 4), although diffuse types of lesions may follow the use of low concentrations of the virus in the inoculum (fig. 5). The symptoms usually require 5 to 7 days to become distinct on the primary leaves, and although the virus moves into the secondary or trifoliate leaves, it rarely causes symptoms on these parts, except a slight stunting such as may be due to a mild allergic reaction of the type mentioned above. On plants nearing maturity, a general re-

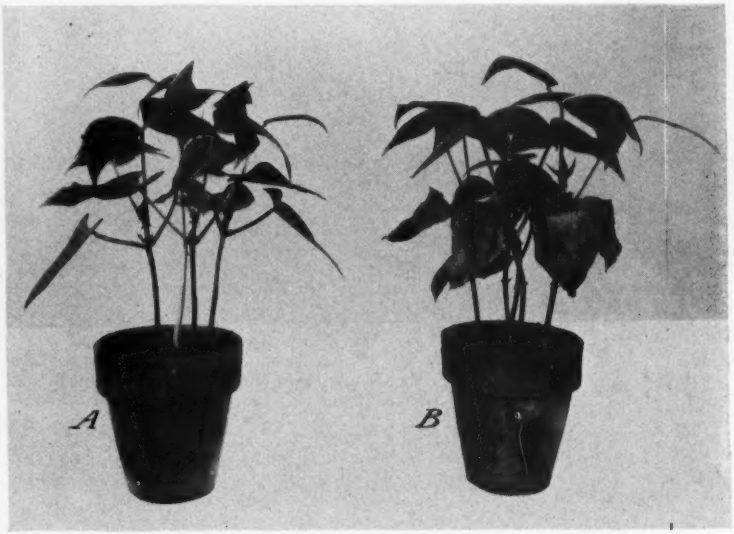

FIGURE 4.—The bean leaf wilt virus: *A*, water inoculated control bean plants; *B*, bean plants inoculated on the primary leaves with the bean wilt virus, resulting in gradual wilting and necrosis of the inoculated leaves but with little effect on the new trifoliate leaves.

tardation of growth vigor is apparent. That the virus is present in the secondary leaves may be shown by transfer.

The properties of the bean leaf wilt virus were determined to be approximately as follows: Thermal death point, 48°–50° C. at 10-minute exposures; tolerance to aging in vitro over 24 hours, in dried leaves over 30 days; tolerance to dilution over 1–1,000.

Preliminary trials indicated that the virus was transmissible by the aphid *Myzus persicae* (Sulz.), small local lesions being formed at the point of feeding by the aphids.

THE BEAN YELLOW NECROSIS VIRUS

The bean yellow necrosis virus was obtained from inoculations to the Refugee bean with young, apparently healthy *Sesbania macrocarpa* Muhl. in two separate trials, but was not secured in several other

trials. The common name "bean yellow necrosis" virus is descriptive of the symptoms on the bean, because both yellowing and necrosis are very marked. The secondary leaves formed soon after the inoculation of the primary leaves are often devoid of green color (fig. 6)

FIGURE 5.—The bean leaf wilt virus: *A*, A primary leaf of the bean inoculated with a low concentration of the bean wilt virus, resulting in a diffuse type of local lesions; *B*, primary leaf from control plant inoculated with water only.

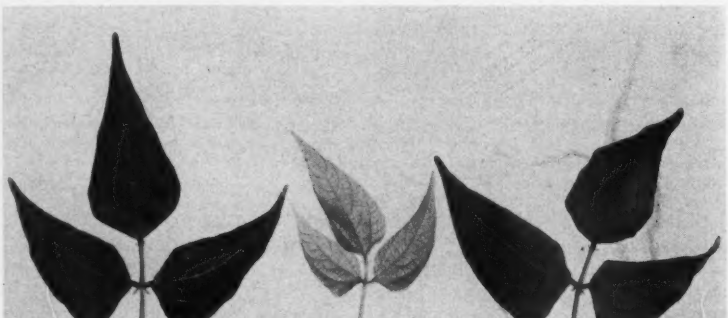

FIGURE 6.—The bean yellow necrosis virus: *A*, Leaf from uninoculated control plant; *B*, almost completely chlorotic leaf from inoculated bean plant; *C*, milder symptoms of chlorosis and vein clearing on right side of trifoliate leaf.

and fail to grow. If young at the time of inoculation, the entire plant may become completely necrotic (fig. 7). Older plants or more resistant varieties may, however, show comparatively mild symptoms, although local necrotic spots on the inoculated primary leaves and mild chlorosis of the secondary leaves are still characteristic. The Refugee bean appears to be relatively susceptible and the Great Northern bean relatively resistant to this virus. Other legume species are susceptible, including the soybean (*Soja max* L.) Piper and the garden pea (*Pisum sativum* L.) (variety Marvel), although the virus appears to be confined to the inoculated leaves of the latter species.

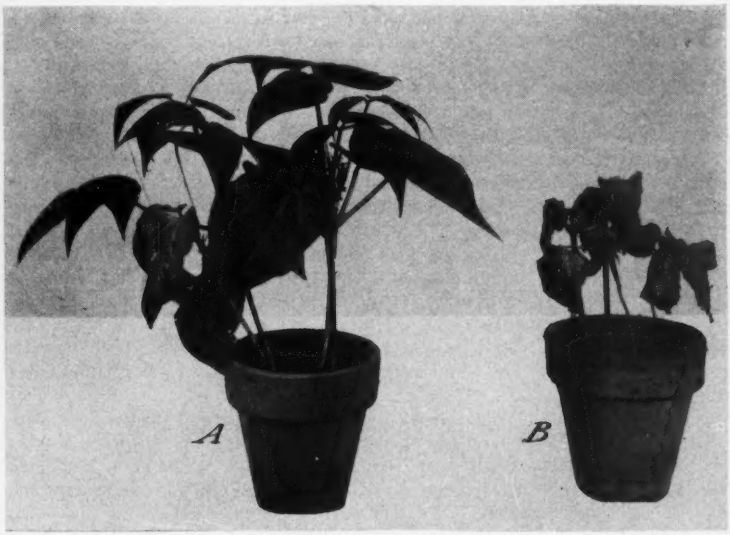

FIGURE 7.—The bean yellow necrosis virus: *A*, Uninoculated control plants, *B*, bean plants inoculated in the primary leaf stage with yellow necrosis virus.

The thermal death point of the bean yellow necrosis virus was found to be 49° C. at a 10-minute exposure. It survives aging in vitro for 48 hours and possibly longer, and in dried leaves for 12 days or longer. It withstands a dilution of 1 to 100,000 and possibly as high as 1 to 500,000. The virus was removed from filtrates by both Mandler and Seitz filters. Cow's milk, which readily inactivates the ordinary tobacco virus, strangely enough does not prevent infection of bean with the bean yellow necrosis virus.

The virus is not transmitted by the aphid *Myzus persicae*, according to results of the present limited trials.

DISCUSSION

The virus diseases of the legumes have been the subject of considerable study in recent years, and the known information on the bean and pea viruses in particular has been summarized by Pierce (*8*) and Zaumeyer (*10*). It has not been possible to make a detailed

laboratory comparison of the viruses described in this paper with the several previously described legume viruses. Only bean virus 1 (ordinary bean mosaic virus) has been compared throughout the tests, largely as a control on transmission and property studies. Judging from the literature, however, the bean leaf wilt virus and the bean yellow necrosis virus appear to have little or no similarity in either symptoms or properties with the earlier described viruses on either bean or pea. The thermal death point of both new viruses is below 50° C., whereas those of the other legume viruses range from 56° to 70°. While the other properties are less consistently different, they are sufficiently so in specific instances to justify the belief that the two viruses isolated from apparently healthy plants of *Lathyrus pusillus* and *Sesbania macrocarpa* have not been previously described.

It is not surprising that such viruses should be found to exist in view of what is known about symptomless carriers of viruses. Nor is it improbable that these same viruses may be responsible for distinctive symptoms on other legume species in nature, occurring only incidentally on *Lathyrus* and *Sesbania*.

A logical proof of the relation of these viruses to the hosts from which they were secured would naturally consist of attempts to transmit the viruses back to the respective hosts from which they were obtained. Unfortunately, these efforts were not carried to a satisfactory conclusion, due in part to the uncertainty that healthy plants were available for inoculation and the unfavorableness of *L. pusillus* as a test plant, and in part to the accidental loss of the yellow necrosis virus during the summer period when the experimental work was in abeyance. The limited trials which were made indicated that the respective hosts could be infected, but that no symptoms were obtainable.

Even though the preexistence of the new viruses described is accepted, and the large number of negative results from the cross-inoculations made between different legume species (119 out of 122 species) are taken into account, a negative conclusion on the viroplasm hypothesis is not yet justified. In the earlier plans for testing the viroplasm theory, it was believed that 100 percent compatibility from any one species would be required to yield positive proof, and that if success were not secured in the first or second attempt at transfer, no further trials were necessary. It now seems quite possible that some unknown circumstance may permit a successful transfer of normal plant protoplasm from one species to another in only rare instances and still be sufficient to account for the number of known viruses. Furthermore, positive results should perhaps not be expected following artificial transfers of plant extract, such as were made in the present experiments. It is more likely that such natural means of inoculation as insect transmission may be first required before the agent concerned becomes adapted to other forms of transmission. Investigation by such methods admittedly would be laborious.

The challenge of the origin of viruses is, however, a goal worthy of unusual efforts. Many keen observers have been led to the belief that new viruses (as contrasted with virus strains which arise as variants) may originate de novo. This is quite as understandable as the assumption that certain of these extremely delicate forms of life have survived eons of time on the evolutionary basis such as applies to the origin of other forms of life. On the assumption of a de novo

origin, the relationship of foreign protoplasm (viroplasm) seems more probable than any other precursor yet described when the ability to reproduce is to be considered.

The reproduction of some protoplasmic constituents (or living protein molecules) of one species in another is not more inconceivable to science than were certain phenomena occurring in symbiosis, parasitism, allergy, graft hybrids, or sexual hybridization before they were first recognized. To be sure, additional difficulties arise in attempting to explain the relation of the viroplasm to insect vectors, but this is no more curious than the remarkable effect of insect incitants on plant tissues, as in plant galls.

The viroplasm hypothesis does not differ greatly from certain other hypotheses which have been promulgated since; e. g., that of Baur (*1*), which assumes some sort of incitant or precursor or abnormal gene (*3*) originating within the host in which the disease first occurred. The viroplasm theory suggests only that this incitant comes originally from a plant closely related to or quite foreign to the host, with the production of abnormal protein molecules, modified cytoplasmic inclusions (*5*), and usually but not always macroscopic symptoms of disease which are transmissible by virtue of the original compatability of certain species in this particular direction.

SUMMARY

Inoculations with extracts from healthy plants to other healthy plants were performed to test the hypothesis that some part of the protoplasm of one species properly introduced into the living cells of another species might find conditions compatible for growth and bring about the abnormality known as a virus disease. This possibility is referred to as the "viroplasm hypothesis."

Extracts from 122 species of legumes representing 50 genera were transferred by the wiping method of inoculation to bean (*Phaseolus vulgaris*) plants, and in some instances to other species. No symptoms were secured from 119 of the species tested. One species, *Lathyrus tingitanus*, regularly yielded symptoms, but these are interpreted as an allergic response. *Lathyrus pusillus* frequently yielded a virus which is new and which is described under the common name of "bean leaf wilt virus." *Sesbania macrocarpa* on two occasions yielded a new virus, described under the common name of "bean yellow necrosis" virus. From present knowledge of symptomless carriers, it seems most likely that the two new viruses preexisted in the hosts and that they did not originate as a result of the transfer of healthy protoplasm to another species.

Although these limited preliminary trials are not regarded as furnishing proof of the validity of the viroplasm hypothesis, they suggest other means of testing the hypothesis and may serve to aid in the interpretation of the experimental results obtained.

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EFFECT OF TRACE ELEMENTS ON GROWTH OF ASPERGILLUS NIGER WITH AMINO ACIDS[1]

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INTRODUCTION

A necessary preliminary to investigation of the effect of deficiencies in trace elements on the growth of *Aspergillus niger* Van Tiegh. with the amino acids is a study of the nutritive properties of these acids. Information in this respect is quite limited and difficult of interpretation because of differences in experimental conditions. Extensive tests, therefore, were made relative to the effects of substituting amino acid nitrogen for inorganic nitrogen prior to the study of trace-element omission. The evidence concerning participation of trace elements in enzymatic deaminations rendered it probable that their omission from the nutrient solution containing amino acids would lead to specially marked decreases in yield. These specific growth effects would be expected to vary with the amino acid and be in agreement with the results obtained on enzyme activation. Modifications in nutrition should, on this basis, be of help also in identifying unsuspected trace-element requirements when specific needs are emphasized in this manner. This procedure has already proved successful with molybdenum (*22*)[3] and scandium (*23*).

The investigations reported here were planned with a view to obtaining information in regard to the process by which amino acids are synthesized in the plant.

Little has been published concerning the mechanism of amino acid synthesis. Kossowicz (*13*) has suggested that nitrogen enters organic combination as nitrate. Baly, Heilbron, and Stern (*2*) consider this process to be one of condensation with formaldehyde to form hydroxamic acid. Lemoigne, Monguillon, and Desveaux (*14*) have attempted to demonstrate that it is hydroxylamine that first enters organic combination. This theory has been strengthened recently by the work of Virtanen and Laine (*33*), who consider that nitrogen fixation by *Rhizobium* occurs through formation of hydroxylamine and its combination with oxalacetic acid to form the oxime of *l*-aspartic acid. The writer (*24*) has pointed out some objections to the hydroxylamine and nitrite theories that would seem to favor the ammonia theory of amino acid synthesis. The ammonia theory has been modified by Björkstén (*5*) to include the intermediate formation of an acid amide, whose condensation with acrylic acid and its homologues results in amino acid formation subsequently. Still another suggestion is that

[1] Received for publication August 22, 1941.
[2] The author wishes to express his appreciation to Dr. Arnold Kent Balls and Dr. D. Breese Jones, of the Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, for their kindness in reading the manuscript before submittal for publication.
[3] Italic numbers in parentheses refer to Literature Cited, p. 473.

the amino acids are formed through decomposition of a hexose-ammonia complex.

It would seem quite certain that nitrogen metabolism in the plant is by no means static, but that synthesis of amino acids is accompanied by a simultaneous break-down of proteins already formed (*7*). Whether this occurs through complete decomposition of specific proteins or only of specific components of these proteins is as yet not clear. This process would seem to take place during growth as well as where maintenance only is concerned. The isotope technique employed by Vickery and associates (*32*) would seem well adapted to the solution of this and similar problems.

Information on the amino acids synthesized by *Aspergillus niger* is incomplete, since few investigators have concerned themselves with this subject. Abderhalden and Rona (*1*) were able to isolate and identify *d*-alanine, *l*-aspartic acid, *d*-glutamic acid, glycine, and *l*-leucine. Vorbrodt (*34*) reported the presence of tyrosine and probably phenylalanine and proline. Skinner (*21*) obtained a test for tryptophane. It seems likely that many, if not all, of the other known amino acids will also be found on further investigation. Takata (*29*), for example, reports cystine, arginine, histidine, and lysine to be present in *A. oryzae*, a form related to *A. niger*.

Previous investigations with *Aspergillus* have disclosed that inorganic nitrogen is brought into organic combination by this fungus as ammonia (*24*) and inorganic sulfur as sulfinic (sulfoxylic) acid (*26*). The organic compounds initially formed are presumably amino acids, since it has long been known that certain of these may serve as sole sources of nitrogen supply. In addition the writer (*26*) has shown that cysteine, cystine, or methionine may be used to fully replace sulfate sulfur. Whether all amino acids are capable of serving as sole sources of nitrogen for *Aspergillus* is unknown.

Aspergillus is able to produce its amino acids from ammonium salts and glucose indefinitely, though it is able also to use a wide variety of organic nitrogen compounds (*8*, *30*, *31*). Nutrition studies on amino acids with *Aspergillus* are in effect tests to learn the extent to which reutilization of these anabolites may occur. According to Butkewitsch (*6*) and Tamiya and Usami (*30*), utilization of amino acid nitrogen is preceded by deamination. Gorter (*10*) was unable, however, to detect the presence of specific deaminases and concluded deamination takes place under the influence of cell respiratory enzymes.

The tentative working hypothesis was adopted that such amino acids as are directly synthesized from hexose and ammonia would be most readily assimilable as general sources of nitrogen and of carbon. These first-formed and therefore "primary amino acids" are considered the probable precursors of the other and therefore "derived amino acids." Since the latter, according to this view, would be produced through a series of more or less complicated transformations, their assimilation would probably be more complex and therefore slower. That is to say, those amino acids closest in composition to the forms first synthesized by the fungus should prove most suitable as general sources of supply. The truth or falsity of this concept of amino acid synthesis will be considered in connection with the experimental data as it is presented.

The primary amino acids would be those formed through amination of carbon chains resulting from oxidative degradation of the sugar molecule. The extent, therefore, to which the carbon structure of the amino acids may be reutilized should serve to give some indication concerning the process of sugar fragmentation. This would be particularly the case in the event that a single amino acid is the precursor to all others (*33*).

Most investigators will probably agree that those amino acids containing the phenyl or other cyclic groups or branched carbon chains do not originate as the result of a primary synthesis from hexose. It may be anticipated, therefore, that amino acids containing these components should prove to be poor sources of nitrogen and carbon and fall into the group of derived acids.

It is certain that not all organisms can be satisfied in their nitrogen requirements by ammonium salts. In a summary by Rose (*19*) it is reported that rats are unable to synthesize *d*-valine, *l*-leucine, *d*-isoleucine, *d*-threonine, *d*-lysine, *l*-tryptophane, *l*-histidine, *l*-phenylalanine, and *l*-methionine. These, together with *d*-arginine, are considered by him to be indispensable for maximum normal growth of the rat. Either isomer of tryptophane, histidine, phenylalanine, and methionine is satisfactory for growth. Inability of the rat to synthesize these amino acids would seem due in some instances to an incapacity for introducing the amino group or forming the appropriate hydroxy or keto carbon structure (*20, p. 992*). So far it has been possible to replace leucine, isoleucine, tryptophane, histidine, and phenylalanine individually with appropriate carbon compounds without interfering with growth. Nevertheless, lysine could not be replaced in this manner.

Mueller and Kapnick (*16*) have compared their results on amino acid requirements of diphtheria bacteria with those of Fildes and Richardson (*9*) on the requirements of *Clostridium sporogenes*. Diphtheria strain "HY" required *valine*,[4] *methionine*, *tryptophane*, *histidine*, glycine, cystine, and glutamic acid. Diphtheria strain "P–W No. 8" required *valine*, *leucine*, *methionine*, cystine, and glutamic acid. *C. sporogenes* required *leucine*,[4] *tryptophane*, *phenylalanine*, *histidine*, *methionine*, *valine*, tyrosine, *arginine*, and cystine. To quote Fildes and Richardson (*9*), "It is interesting to note that these amino acids are almost the same as those involved in animal nutrition."

These data on amino acid nutrition of bacteria are of special interest, since these organisms have been found susceptible of training and adaptation to utilization of ammonium nitrogen, and vice versa. Knight (*12*) has included an extensive review of this subject in his book.

According to Butkewitsch (*6*) the first step in utilization of amino acid nitrogen by *Aspergillus* takes place through deamination. The types of deamination known to occur with micro-organisms have been summarized by Stephenson (*28*) in her book on bacterial metabolism. Experiments by Tamiya and Usami (*30*) with *A. oryzae* led to similar results on deamination with (*isoleucine*),[5] (*arginine*), (serine, *leucine*, glutamic acid, *tryptophane*, proline), (glycine, alanine, *valine*, aspartic acid), (*phenylalanine*, *histidine*), and (*lysine*). Urea was formed in

[4] The compounds italicized here and elsewhere in this paper are indispensable in the nutrition of the rat.

[5] The groups in parentheses are arranged in the order of decreasing growth when used as complete sources of both carbon and nitrogen.

addition to ammonia with arginine, in a manner presumably analogous to that in which it is produced in the animal.

The order specified for efficiency in amino acid utilization by Tamiya and Usami is based on supply levels ranging from N/3 to N/23. Moreover, no provision was made to insure an adequate supply of trace elements. It can be anticipated, therefore, that the order of relative utility determined for *Aspergillus oryzae* will undergo some revision in the future.

Assimilation of amino acid nitrogen by yeast was found by Nielsen (*17*) to be best with glycine, alanine, tyrosine, *leucine*, *isoleucine*, aspartic acid, glutamic acid, and proline. The carbon source was sucrose, supplemented by traces of beer wort. Among the poorest sources of nitrogen supply were *histidine*, *lysine*, and hydroxyproline. Another study by Nielsen (*18*), this time with *Rhizobium*, gave best growth at a nitrogen level of 0.002 molar with glycine, alanine, cysteine, aspartic acid, glutamic acid, *arginine*, *histidine*, ornithine, and *lysine*. Racemic amino acids were employed in double concentration. Sucrose was used to supply carbon, and was supplemented with biotin.

Beaumont and associates (*3*) were unable to obtain good growth of Havana tobacco when grown under aseptic conditions in water culture with alanine, glycine, *histidine*, *leucine*, *phenylalanine*, tyrosine, aspartic acid, glutamic acid, *arginine*, *lysine*, and proline. Asparagine and cystine were usable but inferior to inorganic nitrogen or urea.

The amino acids essential for growth of the heterofermentative lactic acid bacteria (*36*) are *valine*, *methionine*, *threonine*, *phenylalanine*, *arginine*, *lysine*, *leucine* or *isoleucine*, tyrosine, alanine, cystine, glutamic acid, serine, and aspartic acid. *Tryptophane* was essential for one strain.

EXPERIMENTAL METHODS

Aspergillus niger Van Tiegh. (No. 4247)[6] was grown for 4 days at 35° C. in each instance. The nutrient solution used contained the following compounds per liter: Sucrose, 50 gm.; ammonium nitrate, 1.90 gm.; dipotassium phosphate, 0.35 gm.; and magnesium sulfate ($7H_2O$), 0.25 gm. Iron, zinc, copper, manganese, molybdenum, and gallium were added in concentrations of 0.30, 0.30, 0.075, 0.075, 0.02, and 0.02 mg. per liter, respectively. Water used in the preparation of this solution was redistilled in quartz. The 200-cc. Erlenmeyer culture flasks were of transparent quartz as were all other laboratory ware used. Pyrex Erlenmeyer flasks were used for work not dealing with trace elements. Each culture contained 50 cc. of nutrient solution. Further details of technique will be found in a previous publication (*22*).

Trial of nitrogen utilization of amino acids was made by substituting the amino acid for ammonium nitrate in the above formula without alteration in the nitrogen content of the solution. The nitrogen level in all cases was 665 mg. per liter or 33.25 mg. per culture, which is capable of giving a yield of 1,100–1,300 mg. per culture of 50 cc. under the conditions used.

Estimation of carbon utilization could not be made at the carbon level (50 gm. sucrose per liter) necessary for maximum yield, in view of the expense for these quantities of amino acids. Therefore the amino acids were used at a level of 10 gm. per liter. Results on

[6] Collection of Dr. Charles Thom, Division of Soil Microbiology, Bureau of Plant Industry, U. S. Department of Agriculture.

carbon assimilation are tabulated in terms of grams of yield per gram of carbon available in the substrate. The carbon-utilization factor for sucrose is 1.17 gm. of yield per gram of carbon. These values are growth rates and not absolute values on efficiency in conversion. The latter would necessarily be based on carbon actually assimilated and not on carbon supplied.

RESULTS

UTILIZATION OF AMINO ACID NITROGEN

Complete replacement of inorganic nitrogen by amino acid nitrogen (table 1) took place with alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, and proline. These, together with ornithine (table 2), are by definition, therefore, the first-formed or primary amino acids for *Aspergillus*. Imino nitrogen of arginine, hydroxyproline, and proline was employed as effectively as amino nitrogen in the other acids. Moderate utilization of nitrogen occurred with serine, threonine, and tryptophane, corresponding roughly to 50-percent assimilation (*27*). Growth was still poorer with isoleucine, leucine, methionine, phenylalanine, tyrosine, and valine; and very poor with the remainder of the natural amino acids. These results are believed to bear no relation to isomerism, nor to form of nitrogen union. Repetition of the trials, with those amino acids (particularly serine and threonine) employed as racemic mixtures, would be required to prove this opinion. Except for hydroxyproline and proline, which contain the readily opened pyrrolidene ring, none of the amino acids containing a cyclic group or branched carbon chain fully replaced inorganic nitrogen.

TABLE 1.—*Growth attained by Aspergillus niger with amino acid nitrogen (665 mg. per liter) after 4 days at 35° C.*

Amino acid	Yield	Sporulation [1]	Acidity		Starch in mycelium [2]
			Initial	At harvest	
	Mg.		*pH*	*pH*	
dl-α-Alanine	1,115.8	10	6.79	2.06	0
d-Arginine hydrochloride	1,252.4	10	6.81	1.83	2
l-Aspartic acid	1,325.1	10	2.88	2.14	0
l-Cystine	71.0	1		2.25	0
d-Glutamic acid	1,292.8	10	3.18	2.04	1
Glycine	1,234.8	10	6.35	1.99	0
l-Histidine dihydrochloride	120.9	4	2.08	1.93	0
l-Hydroxyproline	1,147.4	10		2.17	1
l-Iodogorgoic acid	.0	0			
dl-Isoleucine	246.2	4		2.88	0
l-Leucine	373.6	6	6.49	2.87	0
d-Lysine dihydrochloride	16.2	0	1.99	1.97	1
dl-Methionine	437.0	7	7.44	2.85	0
dl-Norleucine	113.8	1		2.96	0
dl-Phenylalanine	370.8	4		2.78	2
l-Proline	1,110.7	10		1.93	2
dl-Serine	608.6	7		2.60	0
dl-Threonine	647.4	6		2.30	0
l-Tryptophane	636.3	6		2.61	1
l—Tyrosine	196.1	10		2.85	0
dl-Valine	291.8	1		2.78	0
Cysteine hydrochloride	12.2	0	6.28	5.96	3*
β-Alanine	18.7	4		5.33	0
Lanthionine [3]	92.3	3		2.72	0
Cystine, optically inactive [3]	98.6	1		2.47	3*

[1] Sporulation is rated from 0 (sterile) to 10 (black with spores). In some instances both black and brown (bb) or brown (br) spores were present, Where no notation is made, the spores were the normal black.

[2] Quantity of starch present in the mycelial felts is based on a scale of 0 (none) to 5 (maximum). An immediate blue color with N/20 iodine is indicated by an asterisk (*). When starch was also present in the substrate it is given in parentheses on the same scale

[3] Obtained through the kindness of Dr. D. B. Jones and Dr. M. J. Horn, of the Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture.

TABLE 2.—*Growth attained by Aspergillus niger with miscellaneous nitrogen compounds (665 mg. nitrogen per liter) after 4 days at 35° C.*

Compound	Yield	Sporulation [1]	Acidity		Starch in mycelium [2]
			Initial	At harvest	
	Mg.		*pH*	*pH*	
dl-β,β-Phenylalanine	2.7	0		3.67	0
S-benzylcysteine [3]	63.5	2			0
α-Amino-*n*-butyric acid	605.2	4		3.32	0
β-Amino-*n*-butyric acid	16.2	4		3.38	0
α-Amino-isobutyric acid	.6	0		3.67	0
ε-Amino-*n*-caproic acid	8.0	0		4.88	0
Taurine	75.8	2, br	7.23	2.78	0
d-Glucosamine hydrochloride	446.5	10		1.63	2 (4)
dl-Homomethionine [4]	1.0	0	7.23	3.52	0
Cysteic acid [5]	17.6	2		1.68	0 (1)
Cysteine disulfoxide [5]	81.6	0	7.23	1.89	5 * (1)
β-Hydroxyethylamine [6]	.0	0	7.23		
N-Phenylglycine	.6	0	7.23	5.05	0
dl-Benzoylalanine	346.2	4	7.23	2.72	0
dl-α-Aminophenylacetic acid	155.0	2	7.23	2.53	0
Sarcosine	208.5	10	7.23	2.53	0
dl-Alanine ethyl ester hydrochloride [7]	228.0	4	7.23	1.82	4 (3)
Glycine ethyl ester hydrochloride [7]	531.0	8	7.23	1.58	0
dl-α-Amino-*n*-valeric acid	80.9	2	7.23	3.00	0
δ-Amino-*n*-valeric acid hydrochloride	181.0	6	7.23	1.73	0
d-Ornithine hydrochloride	1,017.3	10	7.23	1.47	5*
dl-α-Amino-α-methylbutyric acid	1.4	0	7.25	3.37	0
Creatine	1.6	0	7.23	4.35	0
Ethylamine hydrochloride	21.8	1	7.23		0
Methyleneamineacetonitrile	.0	0	7.25		0

[1] See table 1, footnote 1.
[2] See table 1, footnote 2.
[3] Obtained through the kindness of Dr. Ben H. Nicolet, of the Bureau of Dairy Industry, U. S. Department of Agriculture.
[4] Obtained through the kindness of Prof. Vincent du Vigneaud, Cornell University Medical School, New York, N. Y. Yield with 50 mg. (instead of 380 mg.) of *dl*-homomethionine per culture. At a sulfur level of 25 mg. per liter, the yield was only 8.0 mg. Therefore utilization of both nitrogen and sulfur was quite poor.
[5] Obtained through the kindness of Dr. Grace Medes, of the Lankenau Hospital, Philadelphia, Pa.
[6] Growth takes place on β-hydroxyethylamine if the substrate is strongly acidified with sulfuric acid prior to sterilization. Starch formation in the mycelial felts would indicate the main product of decomposition under these conditions to be ammonia.
[7] The ethyl esters of glycine and alanine gave variable results but never greater than that equivalent to a 50-percent utilization. Variations are attributed to differences in degree of hydrolysis.

The amino acids capable of fully replacing inorganic nitrogen with *Aspergillus* are all among those classified by investigators of animal physiology as glycogenic (*20*), i. e., capable of conversion into glucose by animals. None are ketogenic (*20*), or acetone precursors. In no instance did an amino acid incapable of synthesis by the rat serve to fully replace inorganic nitrogen with *Aspergillus*. Primary amino acids, or those capable of complete reutilization by *Aspergillus*, are also those capable of synthesis by the rat. Those classified as indispensable amino acids for the rat, except possibly arginine, fall into the derived amino acid group with *A. niger*.

Attention is called to the enhanced production of starch by cysteine even when practically no growth is made. It would be of interest to learn whether cysteine plays a specific role in this process or whether its action is indirect.

None of the sulfur derivatives of the amino acids were capable of providing appreciable assimilable nitrogen with the exception of methionine (tables 1 and 2). The cause of this failure does not seem related to the state of oxidation of the contained sulfur inasmuch as the mercapto group in cysteine and the sulfonic acid group in cysteic acid were equally effective in rendering nitrogen unassimilable. The marked superiority of methionine as a source of nitrogen, compared

with cysteine (cysteine/alanine=0.011, methionine/α-amino-*n*-butyric acid=0.722), was attributed to the additional $-CH_2-$ group separating sulfur and nitrogen. Homomethionine, which contains two additional $-CH_2-$ groups, proved practically valueless, however, as a source of either sulfur or nitrogen (homomethionine/α-amino-*n*-valeric acid=0.110). Substitution for mercapto hydrogen did not increase nitrogen assimilability (S-benzylcysteine).

Full utilization of amino acid nitrogen in the compounds listed in table 2 occurred only with ornithine. Approximately 50-percent utilization took place with *dl*-α-amino-*n*-butyric acid and *d*-glucosamine hydrochloride. Amino nitrogen in the beta position or in caproic acid (α-NH_2, ε-NH_2, α, ε-NH_2) was unassimilable. Neither α-amino nor δ-amino nitrogen of *n*-valeric acid could be used, whereas ornithine (α, δ-NH_2) gave maximum growth. Substitutions for amino hydrogen and carboxyl hydrogen gave decreased nitrogen utilization. Reduction of an acid group (−COOH) to an alcohol group (−OH), as, for example, glycine to β-amino ethyl alcohol, prevented nitrogen assimilation. These results are discussed more fully in the section dealing with enzyme action.

The relative unavailability of nitrogen in the derived amino acids was attributed in the discussion of table 1 to the difficulty with which they are deaminated. The possibility exists, however, that toxicity might be a factor in these responses. In order to eliminate this factor as completely as possible, the experiments were repeated with the derived amino acids in the presence of ammonium nitrate. Since maximum yields are given by ammonium nitrate in concentrations much higher than those employed in the standard solution, it was considered that growth at double the nitrogen level should also be maximum. Table 3 gives the data for yields at a nitrogen level of 1,330 mg. per liter, of which one-half was in the form of ammonium nitrate and the other half an amino acid.

TABLE 3.—*Growth attained by Aspergillus niger after 4 days at 35° C. when supplied with 665 mg. of nitrogen as ammonium nitrate and an equal quantity as derived or synthetic amino acid*

Compound	Yield per 2.5 gm. sucrose	Sporulation [1]	Starch in mycelium [2]
	Mg.		
Ammonium nitrate	1,240.0	2	5*(5)
β-Alanine	1,157.0	10	0
dl-α-Amino-*n*-butyric acid	1,203.6	10	3
β-Amino-*n*-butyric acid	735.6	10	0
dl-α-Amino-isobutyric acid	785.2	6	0
ε-Amino-*n*-caproic acid	705.8	10	0
Cysteine hydrochloride	257.6	0	5*
l-Cystine	1,151.8	10	1
l-Histidine dihydrochloride	1,169.9	2	3(E)
dl-Isoleucine	892.2	4	0
l-Leucine	827.2	10	1
d-Lysine dihydrochloride	1,263.8	4	2(2)
dl-Methionine	919.8	9	0
dl-Norleucine	1,001.8	10	0
dl-Phenylalanine	1,204.6	9	0
dl-β, β-Phenylalanine	1,047.1	10	1
dl-Serine	1,154.3	6	0
dl-Threonine	1,274.2	10	0
l-Tryptophane	1,195.7	7	0
l-Tyrosine	1,247.7	8	1
dl-Valine	1,104.3	8	0

[1] See table 1, footnote 1.
[2] See table 1, footnote 2. A red coloration with iodine solution is indicated by "E," abbreviation for erythrodextrin.

Under these conditions the only amino acids of natural occurrence which did not give maximum yields were cysteine, isoleucine, leucine, and possibly methionine. However, these amino acids gave greater yields in the presence of ammonium nitrate than when used alone. The greater number of derived amino acids were nontoxic at the nitrogen levels employed. The toxicity exhibited by the above-mentioned amino acids is due perhaps to enzyme inactivation through permanent combination with aminases required for introduction of nitrogen into other amino acids. Cysteine had an especially marked action in preventing synthesis of amino acids from ammonia.

The effect of cysteine on the utilization of amino acids is shown in table 4. Its presence led to a diminution in yield on a milligram basis, particularly with the primary amino acids alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, and proline. This result is in conformity with but no proof of the assumption that cysteine combines with the enzyme responsible for both the liberation of amino nitrogen supplied and the subsequent introduction of the ammonia produced to form new amino acids.

TABLE 4.—*Sporulation and yield of Aspergillus niger after 4 days at 35° C. when grown in the presence of 665 mg. of nitrogen as cysteine hydrochloride or of glycerol as carbon source*

Amino acid added in quantity equivalent to 665 mg. of nitrogen per liter	Cysteine hydrochloride 7.48 gm. per liter [1]		Glycerol as carbon source	
	Sporulation [2]	Yield	Sporulation [2]	Yield
		Mg.		*Mg.*
dl-Alanine	0	431.1	8	409.8
d-Arginine hydrochloride	0	113.7	8	119.9
l-Aspartic acid	0	404.1	0	259.3
l-Cystine	0	16.5	8	.9
d-Glutamic acid	0	97.8	8	430.5
Glycine	0	265.9	1	8.7
l-Histidine dihydrochloride	0	158.4	8	116.1
l-Hydroxyproline	0	31.1	4	14.2
dl-Isoleucine	0	45.7	0	2.1
l-Leucine	0	86.1	6	334.7
d-Lysine dihydrochloride	0	89.4	1	23.9
dl-Methionine	0	34.7	4	27.6
dl-Norleucine	0	79.6	1	5.4
dl-Phenylalanine	0	77.5	0	8.3
l-Proline	0	352.9	10	925.5
dl-Serine	0	99.5	1	9.7
dl-Threonine	0	52.8	1	2.1
l-Tryptophane	0	28.7	1	5.4
l-Tyrosine	0	19.6	2	22.8
dl-Valine	0	202.0	0	6.8
Equal quantities of nitrogen as: [3]				
l-Cystine+glycine	10	1,128.9		
l-Cystine+*dl*-alanine	10	1,101.6		
l-Cystine+*d*-glutamic acid	10	1,301.6		
d-Lysine+glycine	10	1,315.9		
d-Lysine+*dl*-alanine	10	1,270.0		
d-Lysine+*d*-glutamic acid	10	1,567.1		

[1] All cultures containing 50 percent cysteine nitrogen gave starch tests equivalent to 5* (5).
[2] See table 1, footnote 1.
[3] These data indicate *l*-cystine inhibits growth only slightly whereas *d*-lysine does so not at all.

Assimilation of amino acid nitrogen when glycerol was substituted for sucrose is of interest from several points of view. Growth of *Aspergillus* on glycerol is very poor (10–400 mg.) with inorganic nitrogen. This carbon source seems most suitable for studying the effect of scandium (*23*) on yields, though inability to obtain maximum

yields with glycerol has proved a serious hindrance. The data would indicate that approximately maximum growth may be obtained on glycerol when nitrogen is supplied as proline.

Yields obtained in the presence of glycerol differ from those similarly obtained with amino acid nitrogen when sucrose is used. Growth was greatest with proline, glutamic acid, alanine, leucine, and aspartic acid, in the order named. Arginine, glycine, and hydroxyproline gave much poorer results than with sucrose, while with leucine and histidine the yields were the same. The reason for these responses is ascribed to the effect of the carbon residues of the amino acids, and the effects of carbon supplementation or admixtures in carbon supply (*23*). Greater weight must therefore be placed on the results of amino acid addition with a good source of carbon (sucrose) than with a poor one (glycerol).

Slight decreases in utilization of glycine, alanine, and glutamic acid are also brought about by *l*-cystine, but not by *d*-lysine. Assimilability of other amino acids on addition of cystine was not investigated.

AMINO ACID STRUCTURE AND ENZYMATIC DEAMINATION

A compilation of the data obtained with glycine and alanine, some of their derivatives, and a few compounds of closely similar structure will be found in table 5. Care has been exercised to omit from each series any closely related amino acids of natural origin possibly subject to the influence of a different enzyme. Exceptions to this rule are safe only if the substances are completely unassimilable. Comparisons could of course be made on a broader basis, if it were definitely known that glycine and alanine reacted with the same enzyme.

TABLE 5.—*Comparison of variations in assimilability of nitrogen by Aspergillus niger obtained through alteration of the molecular structure of glycine and alanine*

Compound	Formula	Yield	Sporulation [1]
		Mg.	
Glycine	$CH_2(NH_2)CO.OH$	1,234.8	10
Glycine ethyl ester hydrochloride	$CH_2(NH_2.HCl)CO.OC_2H_5$	531.0	8
Sarcosine	$CH_2(NH.CH_3)COOH$	208.3	0
dl-α-minophenylacetic acid	$C_6H_5CH(NH_2)COOH$	155.0	2
N-Phenylglycine	$CH_2(NH.C_6H_5)COOH$	.6	0
β-Aminoethyl alcohol	$CH_2(NH_2)CH_2OH$	.0	0
α-Amino-isobutyric acid	$(CH_3)_2C(NH_2)COOH$	.6	0
Methyleneaminoacetonitrile	$CH_2(N{:}CH_2)CN$	.0	0
dl-Alanine	$CH_3CH(NH_2)COOH$	1,115.8	10
β-Alanine	$CH_2(NH_2)CH_2COOH$	18.7	4
dl-Alanine ethyl ester hydrochloride	$CH_3CH(NH_2.HCl)COOC_2H_5$	228.0	4
dl-Benzoylalanine	$CH_3CH(NH.COC_6H_5)COOH$	346.2	4
dl-β-Amino-β-phenylalanine	$C_6H_5CH(NH_2)CH_2COOH$	2.7	0
Cysteine hydrochloride	$HS.CH_2CH(NH_2.HCl)COOH$	12.2	0
S-Benzylcysteine	$C_6H_5CH_2SCH(NH_2)COOH$	63.5	2
Cysteic acid	$HO_3SCH_2CH(NH_2)COOH$	17.6	2
Taurine	$HO_3SCH_2CH_2.NH_2$	75.8	2, br
(*dl*-Serine)	$HOCH_2CH(NH_2)COOH$	608.6	7
(*dl*-α-Amino-*n*-butyric acid)	$CH_3CH_2CH(NH_2)COOH$	605.2	4
(α-Amino-isobutyric acid)	$(CH_3)_2C(NH_2)COOH$	.6	0

[1] See table 1, footnote 1.

Glycine, the simplest of the amino acids, contains three significant groups: Carboxyl, α-amino, and α-hydrogen. Examination of the compounds listed will disclose that esterification of glycine or reduc-

tion of carboxyl inhibits utilization. Complete replacement of α-hydrogen or even partial substitution of amino hydrogen leads to a similar result.

The alanine molecule contains each of the characteristic groupings of glycine, and in addition contains β-hydrogen. It will be seen that responses to modifications in alanine parallel those with glycine. A shift of the amino group from the α to the β carbon atom also destroys assimilability. Most interesting of all, however, are the decreases in utilization brought about by replacements of β-hydrogen.

These responses parallel so closely those obtained in vitro in the study of enzyme action on the peptide (–NHCO–) group (*4*) that there can be little question but that both are fundamentally due to the same intrinsic type of enzymatic mechanism. The difference in results—deamination as compared to opening of the peptide linkage—is probably due to the different arrangement of these forces to insure strain and rearrangement at different points of the substrate molecules.

UTILIZATION OF AMINO ACID CARBON

No information is available concerning the oxidative break-down of sugar that leads to production of α-keto or hydroxy acids presumably required for direct amino acid formation. Even the number of carbon chains employed in amino acid synthesis is unknown as yet, although the data of table 1 would suggest that individual units for each of the known amino acids are unnecessary. Assuming that only the primary amino acids are directly synthesized from degradation products of carbohydrate, the number required would still be six. This would mean separate keto or hydroxy acids for formation of alanine, arginine (ornithine), aspartic acid, glutamic acid, glycine, and proline (hydroxyproline), unless interconversions occur among these also.

If any basis exists for the assumption that only a few of the amino acids are of primary origin, these same acids should serve best as sources of carbon. Moreover, the extent to which the carbon of the sugar is replaceable by amino acid carbon should serve as a measure of the proportion of carbohydrate carbon employed for formation of amino acids. The rapid appearance in the substrate of ammonia when nitrates are used is evidence that larger quantities are formed than appear in the cellular material. This decomposition of amino acids is without doubt a necessary accompaniment of growth.

The data of table 6 reveal, in fact, that only certain of the primary amino acids are suitable to any degree as sources of carbon. These are arginine, aspartic acid, glutamic acid, ornithine, and proline. Alanine, glycine, and hydroxyproline were quite ineffective for this purpose. Moreover, the reality of the proposed classification of the amino acids into primary and derived groups on the basis of their suitability as sources of nitrogen is substantiated by their behavior as sources of carbon. The low efficiencies in carbon utilization as compared to sucrose (1.17) are interpreted to mean that several of the primary amino acids are formed directly from hexose and ammonia and the balance by subsequent simple interconversions from these. This point will be discussed shortly.

As previously mentioned, the formation of a single primary amino acid as precursor to all others would be indicated by its effectiveness

when substituted for sucrose. If several are so formed as precursors, their use in admixture should also lead to high replaceability of sucrose. Replaceability need not be complete, however, since a certain proportion of sucrose is employed for purposes other than formation of amino acids. No certainty exists that amino acids can serve for effective replacement of sucrose used in respiration and fermentation.

A mixture of glutamic acid, ornithine, and proline gave a yield of 0.82 gm. per gram of carbon. This was the maximum factor obtained with any amino acid mixture. Aspartic acid could be substituted for glutamic and arginine for ornithine. This value could not be increased by addition of glycine, alanine, hydroxyproline, or leucine. An interesting and important feature of these results is the fact that glutamic acid, ornithine, and proline are all α, δ-substituted valeric acid derivatives. Interconversion probably accounts for equivalence between glutamic and aspartic acids (*15*), and the formation of the other primary acids (*35*). Admixture studies therefore also confirm the hypothesis that the amino acids first formed possess maximum assimilability.

TABLE 6.—*Sporulation and relative carbon assimilation of amino acids by Aspergillus niger after 4 days' growth at 35° C.*

[Sucrose = 1.17]

Compound	As source of carbon		As source of carbon and nitrogen	
	Sporulation[1]	Yield per gram of carbon	Sporulation	Yield per gram of carbon
		Gm.		*Gm.*
dl-Alanine	1	0.02	2	0.09
d-Arginine hydrochloride	0	.10	0	.02
l-Aspartic acid	4	.21	2	.23
l-Cystine	0	.00	0	.00
d-Glutamic acid	4	.23	4	.27
Glycine	0	.00	0	.00
l-Histidine dihydrochloride	0	.00	0	.02
l-Hydroxyproline	1	.06	2	.07
dl-Isoleucine	0	.00	0	.00
l-Leucine	0	.01	0	.01
d-Lysine dihydrochloride	0	.00	0	.06
dl-Methionine	0	.00	0	.00
dl-Norleucine	0	.00	0	.00
dl-β-Phenylalanine	0	.00	0	.00
l-Proline	2, br	[2].34	6, br	.15
dl-Serine	0	.00	0	.00
dl-Threonine	0	.00	0	.00
l-Tryptophane	0	.00	0	.00
l-Tyrosine	0	.00	0	.00
dl-Valine	0	.00	0	.00
Creatine	0	.00		
Cysteine hydrochloride	0	.00	0	.00
α-Amino-*n*-butyric acid	0	.00	0	.00
α-Amino-isobutryic acid	0	.00		
ϵ-Amino-*n*-caproic acid	0	.00	0	.00
dl-α-Amino-*n*-caprylic acid	0	.00	0	.00
d-Glucosamine hydrochloride	1	.03	2	.07
Asparagine	1	.02	0	.01
dl-α-Amino-*n*-valeric acid	0	.00	0	.00
d-Amino-*n*-valeric acid	0	.00	0	.00
d-Ornithine dihydrochloride	1	.10	2	.07

[1] See table 1, footnote 1.
[2] Another sample gave a value of 0.22.

ASSIMILABILITY AND MOLECULAR CONFIGURATION

In view of Gorter's (*10*) evidence that specific deaminases do not enter into these reactions, it is of interest to summarize some of the biological data from the chemical viewpoint. Table 7 shows data on the effect of the length of the carbon chain on assimilability of the α-amino monocarboxylic acids by *Aspergillus niger*. Availability as nitrogen supply decreases rapidly with increased length of carbon chain. Utilization of these acids as sources of carbon is practically nil. The introduction of an amino group or oxygen at the terminal carbon atoms of these acids increases both nitrogen and carbon utilization considerably (aspartic and glutamic acids, proline, ornithine). Lysine is an exception, but α-amino adipic acid may prove slightly more available.

TABLE 7.—*Effect of length of carbon chain on assimilability of α-amino monocarboxylic acids by Aspergillus niger*

Amino acid	Carbon chain	Yield as nitrogen source	Yield per gram carbon
		Milligrams	*Gram*
Glycine	2	1,234.8	0.00
Alanine	3	1,115.8	.02
α-Amino-*n*-butyric acid	4	605.2	.00
α-Amino-*n*-valeric acid	5	80.9	.00
Norleucine	6	113.8	.00
α-Amino-*n*-caprylic acid	7	----------	.00

TRACE-ELEMENT DEFICIENCIES IN UNPURIFIED SOLUTIONS

The effects of the omission of biologically essential trace elements from the nutrient solution containing amino acid nitrogen are shown in table 8. The amino acids included cover the full range in response by the fungus. Omission of trace elements from the nutrient solution resulted only in minor decreases in growth. This would indicate the presence of relatively large quantities of these elements as impurities or possibly a diminished requirement. Perhaps their purification is not carried to the same degree as with reagent chemicals or it may be that these impurities are retained more tenaciously by amino acids (*11*). Responses with trace-element deficiencies, however, took place with each amino acid that was used.

TABLE 8.—*Effects of deficiencies in trace elements on growth of Aspergillus niger with amino acid nitrogen after 4 days at 35° C.*

Element omitted	Glycine					α-Alanine				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	1,142.9	100.00	1.99	0	10	1,065.3	100.00	2.06	0	10
Fe	1,133.4	99.17	2.59	0	10	998.3	93.71	1.73	0	10
Zn	339.7	29.72	2.63	0	10	530.5	49.80	2.58	0	10
Cu	1,234.8	108.04	2.03	0	10, br	1,115.8	104.74	1.97	0	10, br
Mn	1,092.3	95.57	1.87	0	10	984.4	92.41	2.07	0	10
Mo	1,109.5	97.08	1.97	0	10	1,089.7	102.29	2.42	0	10
Ga	1,152.8	100.87	2.00	0	10	1,086.0	101.94	2.01	0	10
Max. [3]	1,234.8	----------	----------	----------	----------	1,115.8	----------	----------	----------	----------
C. U. [4]	----------	49.39	----------	----------	----------	----------	44.63	----------	----------	----------
pH [5]	----------	----------	6.35	----------	----------	----------	----------	6.79	----------	----------

See footnotes at end of table.

TABLE 8.—*Effects of deficiencies in trace elements on growth of Aspergillus niger with amino acid nitrogen after 4 days at 35° C.*—Continued

Element omitted	*d*-Arginine hydrochloride					*l*-Histidine dihydrochloride				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	1,236.1	100.00	1.83	2	10	89.0	100.00	1.93	0	4
Fe	364.1	29.46	2.29	1	6	63.7	71.61	1.92	0	4
Zn	432.2	34.96	2.23	1	8	80.8	90.79	2.08	0	4
Cu	1,241.6	100.44	1.83	0	10	102.9	115.6	1.85	0	4
Mn	1,179.3	95.40	1.80	1	10	33.0	37.08	1.76	3*	4
Mo	1,237.4	100.11	1.81	3	10	73.3	82.35	1.89	0	4
Ga	1,238.0	100.15	1.83	0	10	116.1	130.43	1.84	0	4
Max. [3]	1,252.4					120.9				
C. U. [4]		50.10					4.84			
pH [5]			6.81					2.08		

Element omitted	Cysteine hydrochloride					*dl*-Methionine				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	11.5	100.00	5.96	3*	0	437.0	100.00	2.85	0	7
Fe	.9	7.77	5.95	0	0	333.3	76.27	2.64	0	4
Zn	9.2	79.61	5.98	3*	0	273.1	62.49	2.62	0	9
Cu	8.2	70.87	6.02	3*	0	412.3	94.35	2.61	0	8
Mn	0	0.	6.03	0	0	349.5	79.98	2.67	1	4
Mo	9.3	80.58	6.02	4*	0	431.4	98.72	2.59	0	8
Ga	9.3	80.58	6.01	3*	0	405.9	92.88	2.59	0	8
Max. [3]	12.2					437.0				
C. U. [4]		.49					17.48			
pH [5]			6.28					7.44		

Element omitted	*l*-Aspartic acid					*d*-Glutamic acid				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	1,196.8	100.00	2.14	0	10	1,284.1	100.00	2.04	0	10
Fe	367.2	30.68	2.66	0	4	1,293.5	100.73	2.19	0	10
Zn	210.0	17.55	2.88	0	10	679.9	52.95	2.41	0	10
Cu	1,325.1	110.72	2.27	0	10, br	1,277.7	99.50	2.05	0	10
Mn	979.3	81.83	1.89	1	1	1,298.5	101.12	1.87	2	8
Mo	1,115.3	93.19	2.14	0	10	1,315.7	102.46	1.98	2	10
Ga	1,175.0	98.18	2.19	0	10	1,292.8	100.68	2.01	0	10
Max. [3]	1,325.1					1,315.7				
C. U. [4]		53.00					52.63			
pH [5]			2.88					3.18		

See footnotes at end of table.

TABLE 8.—*Effects of deficiencies in trace elements on growth of Aspergillus niger with amino acid nitrogen after 4 days at 35° C.*—Continued

Element omitted	*l*-Leucine					*d*-Lysine dihydrochloride				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	325.4	100.00	2.87	0	6	14.8	100.00	1.97	1	0
Fe	155.1	47.66	2.95	0	0	14.2	96.06	1.95	1	0
Zn	209.2	64.29	2.94	0	4	13.3	89.76	1.94	0	0
Cu	304.4	93.55	2.81	0	6	13.9	93.70	1.92	1	0
Mn	373.6	114.80	2.71	0	8	16.2	109.45	1.92	1	0
Mo	208.7	64.14	2.99	0	2	14.4	96.85	1.93	0	0
Ga	256.1	78.70	2.87	1	4	14.7	99.21	1.93	1	0
Max. [3]	373.6					16.2				
C. U. [4]		14.94					.65			
pH [5]			6.49					1.99		

[1] See table 1, footnote 2.
[2] See table 1, footnote 1.
[3] Maximum individual yield.
[4] Coefficient of utilization, or yield per 100 gm. of sucrose.
[5] Initial acidity of nutrient solution.

Extent of sporulation and of shifts in acidity varied approximately with the magnitude of growth. Little formation of starch took place in the mycelial felts except after growth on cysteine. Its production occurred in this case despite the relatively low acidities following growth on cysteine. An increased acidity should lead to a further intensification in elaboration of starch (*25*).

TRACE-ELEMENT DEFICIENCIES IN PURIFIED SOLUTIONS

Additional tests to determine the effects of trace-element deficiencies on *Aspergillus niger* when grown with amino acids were carried out after purification with calcium carbonate. The results are tabulated in table 9. Improved results were obtained in many instances after omission of trace elements following nutrient solution purification. The improvements were slight, however, and poorer than those usually obtained when inorganic nitrogen is employed. These results further demonstrate the retention of trace-element impurities by the amino acids. It is possible that retention of impurities by the amino acids is to be associated with the presence of amino nitrogen and the formation of unionized complexes (*11*). Nevertheless it is clear that the trace elements needed for growth with inorganic nitrogen are also required with amino acid nitrogen.

DISCUSSION

An important phase of amino acid nutrition deals with specificity in requirements and the comparative needs of different organisms. Data in the literature would indicate that marked specificity exists with the bacteria and fungi, accompanied by marked variations between species and even strains. Animals, by contrast, do not seem to differ in amino acid requirements. It may well be that further study will reveal greater uniformity to exist among lower forms also. A contrasting variation in technique which may be partly responsible for this situation is the use of complex and effective carbon sources with animals. Lower organisms are usually grown with a single highly purified carbon source.

TABLE 9.—*Effects of deficiencies in trace elements on growth of Aspergillus niger, with amino acid nitrogen in solutions purified with calcium carbonate, after 4 days at 35° C.*

Element omitted	α-Alanine					Glycine				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	1,173.7	100.00	2.57	0	10	1,342.0	100.00	2.61	0	16
Fe	15.5	1.33	3.02	0	2	35.0	2.61	2.95	0	1
Zn	18.6	1.60	2.86	0	2	53.7	4.00	2.85	0	2
Cu	1,008.2	85.90	2.64	1	6, br	1,349.5	100.56	2.58	0	6, br
Mn	644.7	55.32	2.14	2	1	1,445.9	107.74	2.33	0	10
Mo	1,111.5	94.70	2.63	0	10	1,225.5	91.32	2.52	0	10
Ga	1,160.9	98.91	2.61	0	10	1,292.5	96.31	2.50	0	10
Max.[3]	1,219.7					1,459.8				
C. U.[4]		48.79					58.39			
pH [5]			6.85					7.83		

Element omitted	*l*-Aspartic acid					*d*-Glutamic acid				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	643.4	100.00	3.02	0	10	912.4	100.00	2.78	2	10
Fe	17.5	2.72	3.64	0	0	27.5	3.01	3.62	0	1
Zn	43.6	6.78	3.13	0	2	21.3	2.33	3.35	0	1
Cu	619.4	96.27	3.01	0	10	944.5	103.51	2.58	2	10
Mn	614.5	95.51	3.01	0	10	980.5	107.46	2.26	1	10
Mo	626.6	97.39	2.99	0	10	893.2	97.90	2.76	3	10
Ga	663.8	103.17	2.92	0	10	892.0	97.76	2.71	2	10
Max.[3]	663.8					980.5				
C. U.[4]		26.55					39.22			
pH [5]			5.33					5.27		

Element omitted	*l*-Leucine					*dl*-Norleucine				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	473.4	100.00	2.85	0	1	94.5	100.00	2.82	1	0
Fe	67.3	14.27	2.90	0	0	41.9	44.38	2.65	0	0
Zn	220.8	46.28	2.80	0	6	44.7	47.34	2.67	0	0
Cu	546.6	115.40	2.81	0	1	95.3	100.83	2.77	1	0
Mn	650.9	137.74	2.87	0	1	85.0	89.94	2.79	1	0
Mo	558.6	118.90	2.83	0	1	89.3	94.56	2.78	1	0
Ga	539.7	94.80	2.87	0	1	94.6	100.12	2.80	1	0
Max.[3]	546.6					97.0				
C. U.[4]		22.34					3.88			
pH [5]			6.89					6.69		

See footnotes at end of table.

TABLE 9.—*Effects of deficiencies in trace elements on growth of Aspergillus niger, with amino acid nitrogen in solutions purified with calcium carbonate, after 4 days at 35° C.*—Continued

Element omitted	*dl*-Isoleucine					*dl*-Valine				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	156.7	100.00	3.11	0	4	216.4	100.00	3.00	0	2
Fe	8.1	4.54	3.20	0	1	4.7	2.17	3.41	0	2
Zn	22.3	12.45	3.00	0	2	16.5	7.61	3.06	0	0
Cu	166.1	92.87	3.09	0	4	131.8	60.92	3.09	0	1
Mn	145.8	81.52	3.11	0	4	172.4	79.67	3.10	0	2
Mo	184.3	130.48	3.02	0	4	138.9	64.20	3.10	0	2
Ga	198.6	111.02	3.04	0	4	120.0	55.43	3.09	0	2
Max.[3]	261.5					267.0				
C. U.[4]		10.46					10.68			
pH [5]			7.04					7.04		

Element omitted	*dl*-Threonine					*l*-Tryptophane				
	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]	Yield per 2.5 gm. sucrose	Relative yields	Acidity at harvest	Starch in mycelium [1]	Sporulation [2]
	Mg.	*Percent*	*pH*			*Mg.*	*Percent*	*pH*		
None	418.6	100.00	2.87	0	8	493.7	100.00	2.67	4	8
Fe	12.2	2.90	3.07	0	2	18.9	3.82	2.70	0	2
Zn	91.0	21.74	2.75	0	6	234.9	47.58	2.63	0	2
Cu	367.2	87.71	2.82	0	8, br	402.7	81.57	2.76	4	4
Mn	349.8	83.55	2.85	0	8	499.3	101.13	2.44	3	4
Mo	354.3	84.64	2.88	0	8	499.7	101.22	2.75	3	8
Ga	358.3	85.59	2.88	0	8	506.0	102.49	2.63	2	8
Max.[3]	482.0					493.7				
C. U.[4]		19.28					21.67			
pH [5]			6.42					6.53		

[1] See table 1, footnote 2,
[2] See table 1, footnote 1,
[3] Maximum individual yield,
[4] Coefficient of utilization, or yield per 100 gm. of sucrose.
[5] Initial acidity of nutrient solution.

Requirements of *Aspergillus* were found to be somewhat different with glycerol than with sucrose. Other sources of carbon, even in minute traces, can probably cause similar variations in amino acid needs. Data to be published in another connection would indicate that minor differences may arise in "mutants" of *A. niger*, though these do not change the characteristic pattern of the normal strain. Such variations as appear can usually be associated with destructive modifications.

A distinction is necessary between results with those organisms capable and those incapable of assimilating inorganic nitrogen. Patterns of amino acid utilization will differ profoundly. Ammonia users, like *Aspergillus*, will give responses based largely on capacity for digestion, i. e., deamination. Organisms with obligatory amino acid requirements give results based on anabolism, i. e., amination.

Response to amino acid nutrition by plants should serve to render clearer the mechanism of nitrogen storage. It is evident that those amino acids incapable of utilization when supplied from an external source are presumably unsuitable for accumulation as a nitrogen

reserve. On the basis of the responses obtained with *Aspergillus niger*, it would appear that alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, ornithine, and proline may be fully reutilized as general sources of nitrogen supply. Where both nitrogen and carbon reserves are concerned the data would indicate that glutamic acid, aspartic acid, and proline would be most suitable. It is believed to be more than a coincidence that glutamic and aspartic acids are known to function in this way in the green plant in the form of their monoamides (*15*). Availability of reserves in the seedlings important for their initial development would appear to necessitate the inclusion of carbon as well as nitrogen, since the capacity for photosynthesis is not fully developed until later.

Since interconversions of amino acids are known to take place readily, it is not strange to find investigators listing certain amino acids as alternatives. Such a procedure of course does not fully answer the question as to which is actually needed.

The degree of correlation between the results obtained on nitrogen and on carbon availability of the amino acids would strongly indicate the truth of the assumption made concerning a relation between primary origin from hexose and ammonia and rate of assimilability. Deamination or assimilation intact proceeds most readily with these first-formed amino acids, since they are in fact fully equivalent to inorganic nitrogen. Moreover, the carbon structures of these primary amino acids have not been altered appreciably from those of the original products of the oxidative degradation of hexose, to judge from their efficacy as sources of carbon. On the other hand, no real evidence was obtained that the derived amino acids are actually derived from the primary acids. It seems possible that this might be so, since the primary amino acids are fully effective as general sources of nitrogen supply. Proof of this hypothesis would require, however, that evidence be obtained that one or more of these primary amino acids is utilized without deamination.

It could not be determined whether specific quantitative trace-element requirements are associated with utilization of amino acids, nor was it possible to use this procedure in identifying unsuspected mineral requirements. Purification procedures particularly applicable to the amino acids will be necessary to permit of a repetition of these experiments at a higher level of purity. Until this has been accomplished the use of amino acids in trace-element studies should be avoided if possible.

The retention of minute quantities of the biologically essential trace elements, despite the high pH (7.0) of purification, may be found of importance in other studies. It would indicate that organic matter containing nitrogen can aid in maintaining availability of trace elements to the plant over a wide range in acidity. A similar relation may exist between the amino acids of the cell and absorbed trace elements. Amino acid complexes with heavy metals are known to exist (*11*).

Another possible explanation for the poor results on trace elements with amino acid nitrogen is the possibility of a decrease in requirements. This interpretation is unlikely if deamination is a preliminary to utilization as has been asserted. Nevertheless it cannot be eliminated without proof in the event any proportion of the amino acids are utilized as such.

SUMMARY

A survey was made of the assimilability of amino acid nitrogen and carbon with *Aspergillus niger* Van Tiegh. when grown in synthetic nutrient solutions at 35° C. for 4 days. Nitrogen was used at a level of 665 mg. per liter. Nitrogen in alanine, arginine, aspartic acid, glutamic acid, glycine, hydroxyproline, ornithine, and proline proved to be fully equivalent to inorganic nitrogen. Serine, threonine, and tryptophane gave yields corresponding to 50-percent utilization and the balance to far less. None of the amino acids fully effective as sources of nitrogen contained a stable cyclic group or branched carbon chain, nor were they among those incapable of synthesis by the rat. Assimilability of amino acid nitrogen was affected by use of a poor source of carbon supply (glycerol), though the characteristic amino acid utilization pattern was still recognizable.

Cysteine inhibited growth with both inorganic and amino acid nitrogen, and enhanced starch formation. Nitrogen utilization was very poor with cysteine, cystine, and homomethionine, and only fair with methionine. In contrast to the other sulfur- containing amino acids, homomethionine could not serve as a source of sulfur. Availability of cysteine nitrogen was not improved by oxidation of its mercapto group or replacement of mercapto hydrogen.

Only proline, aspartic acid, glutamic acid, ornithine, and arginine were at all effective as sources of carbon, the carbon utilization factors being 0.22, 0.21, 0.23, 0.10, and 0.10 respectively. These were also fully effective as sources of nitrogen. Effectiveness as sources of carbon increased synergistically in admixtures. A mixture of proline, glutamic acid, and ornithine had a carbon utilization factor of 0.82 as compared to a factor of 1.17 with sucrose. Utilization of carbon in this mixture was not increased by the addition of alanine, arginine, aspartic acid, glycine, and hydroxyproline.

Increasing length of the carbon chain in the α-amino monocarboxylic acids was accompanied by a rapid decrease in the assimilability of their nitrogen. Introduction of an amino group at terminal carbon or its oxidation to carboxyl considerably increased assimilability of both nitrogen and carbon in the four- and five-carbon acids.

Alanine, arginine (ornithine), aspartic acid, glycine, glutamic acid, and proline (hydroxyproline) are considered to be the first-formed amino acids in the synthetic process and are therefore called "primary" amino acids. These were considered probable precursors of the other amino acids synthesized by the fungus, which are termed "derived" amino acids. Carbon assimilation studies, however, indicated that α, δ-derivatives of valeric acid (glutamic acid, ornithine, proline) are actually the first of the primary acids formed, the balance being products of relatively simple interconversions.

Growth responses to structural modification in glycine and alanine when used as sources of nitrogen closely paralleled the results of analogous studies in vitro reported for enzymatic action on dipeptides. Nitrogen utilization was found directly dependent on the presence of an alpha amino group and of alpha, beta, and carboxyl hydrogen. Introduction of substituents for alpha, beta, or carboxyl hydrogen inhibited assimilability of nitrogen, as did also reduction of carboxyl or a shift of the amino group to the beta carbon atom.

The trace elements iron, zinc, copper, manganese, molybdenum, and gallium were required with amino acid nitrogen as with inorganic nitrogen. Minute quantities of these elements were tenaciously retained by the amino acids, as compared to inorganic and urea nitrogen, during purification of the nutrient solution with calcium carbonate.

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OCCURRENCE OF THE DWARF-RED CHARACTER IN UPLAND COTTON[1]

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INTRODUCTION

Red coloration in the cotton plant, due to an increase in the anthocyanin pigmentation, is a genetic character that has received wide study. For literature on the inheritance of this character the reader is referred to Carver (*1*),[3] Harland (*2*, *6*), Hutchinson (*8*, *10*), McLendon (*14*), and Ware (*15*, *17*). Hutchinson and Silow (*11*) have listed a multiple allelomorphic series of six types of anthocyanin pigmentation in the Asiatic cottons. A similar allelomorphic series has been established by Harland (*6*) for cottons of the New World group. This latter series, however, does not include any red-plant types occurring in upland cotton, to which belong most of the types studied by investigators (*1*, *13*, *14*, *15*, *17*) in this country. Harland (*6*) states that *Gossypium hirsutum* (upland) red R_1 (*11*) is a duplicate gene to the anthocyanin allelomorphic series R_2 of the New World cottons. Hutchinson, Silow, and Stephens (*12*) have recently reported that the anthocyanin pigmentation of each of the wild species of *G. thurberia*, *G. armourianum*, and *G. aridum* belongs to the R_1 series of *G. hirsutum*. The only members of this series previously reported are red plant and normal green plant.

The dwarf character in cotton appears very infrequently as compared to the red-plant character. Harland (*3*, *4*, *6*) reported the occurrence of "crinkled dwarf" in sea-island cotton. He stated that this mutant is much smaller than and fully recessive to normal sea-island cotton. Crinkled dwarf was thought to occur only in sea-island cotton but has now been reported by Hutchinson and Ghose (*9*) in *Gossypium hirsutum* L. (upland cotton). In this species also the crinkled-dwarf character behaves as a complete recessive to normal. Horlacher and Killough (*7*) produced dwarf cotton plants by means of X-ray treatment of the dry seeds. As yet no inheritance studies have been reported for this type of dwarfing.

In the present paper a new type of red-plant color, together with a new type of dwarfing, in Acala cotton is described, and the results of a study of the inheritance of this character, herein designated as "dwarf-red," are presented.

DESCRIPTION OF THE DWARF-RED CHARACTER

The dwarf-red mutation of Acala cotton originated as a red chimera on a plant of normal height. It was discovered in a planting of

[1] Received for publication August 8, 1941.
[2] The writer is indebted to Dr. R. C. Lindner, of the Bureau of Plant Industry, for making the chlorophyll determinations.
[3] Italic numbers in parentheses refer to Literature Cited, p. 481.

foundation seed at the United States Cotton Field Station, Shafter, Calif. When first observed the mutant plant was 10 inches high, and the first red leaf was about one-half normal size. As the plant developed, the somatic variation proved to be a deep-red band comprising about one-third of the circumference of the main axis. The plant finally grew to a height of about 4 feet. As the height increased, the band of red spiraled around the axis, producing a red fruiting branch wherever it happened to coincide with a node. All nodes on the plant not intersected by the band of red produced normal green vegetation. Attempts to obtain self-pollinated seed from the red portion of the plant were unsuccessful. However, open-pollinated seed from the red fruiting branches was planted the second year to produce a progeny of 17 plants. Twelve of these plants were red throughout and the remaining five were green. The green plants had the appearance of normal Acala in every respect. The occurrence of green plants indicates that all seed produced by the chimera did not possess the factor for the dwarf-red character. The red plants varied considerably in height, but all were much shorter than the normal Acala plants. The variation in height of the red plants is further evidence of the heterozygous nature of the seed produced by the dwarf-red chimera. As will be shown later, the heterozygous dwarf-red is intermediate in height between the homozygous dwarf-red and the normal green. Self-pollinated seed was obtained from all plants of the second year except a very dwarf-red one.

The progenies of eight plants were grown the third year. Three of these progenies were from green plants and were comprised of normal green plants. The other five progenies originated from self-pollinated red plants of the second year. All segregated into three distinct types, dwarf-red, normal green, and a type intermediate in both height and red color. Of these five segregating progenies, three gave an indication of a 1 : 2 : 1 ratio. The data for this segregation are presented in table 1.

TABLE 1.—*Third-year segregation of plant lines originating from a dwarf-red chimera*

Progeny	Dwarf red	Intermediate	Normal green	χ^2	P
RA-3	5	13	7	0.36	0.80+
RA-5	3	11	10	4.25	.10+
RA-10	4	13	8	1.32	.50+
RA-11	2	10	12	9.00	.01+
RA-12	2	9	12	9.78	.01−
Total	16	56	49		

The monohybrid ratio obtained for three progenies is not of great significance, owing to the small numbers involved. The fact that these progenies even approached a monohybrid ratio would show that the seed produced by the chimera was heterozygous, or cross-pollinated with normal green cotton, or both. The deficiency of dwarf-red individuals would indicate that many were too weak to survive. This fact is borne out by observation, as many of this type that did survive were too weak to produce bolls. However, after the

dwarf-red was reduced to homozygosity and crossed with normal green Acala, differential viability did not occur apparently in the recovered dwarf-red segregates of the F_2. This will be noted subsequently.

Dwarf-red Acala in the homozygous condition averages 19 inches in height as contrasted with 48 inches for normal green and 32 inches for the heterozygote. Dwarfness is characterized by a decrease in the length of the internodes, as well as a decrease in the number of nodes. The homozygous dwarf-red Acala averages 23 nodes, the heterozygous dwarf-red 30 nodes, and the normal green 35 nodes per plant.

The coloring of dwarf-red Acala differs from that in other red-plant types of upland cotton. Ware (*15*, *16*, *17*) studied the Winesap variety of cotton and found that this variety in the homozygous condition is a deep red throughout the entire plant, stem, foliage, flowers, and bolls. In contrast to the Winesap variety, dwarf-red Acala has pale cream-colored flowers comparable to those of normal green Acala. In addition, the bolls of dwarf-red Acala are green as contrasted with the red bolls of other red-plant types of cotton. This latter observation caused R. A. Silow, who visited the Shafter, Calif., station in 1939, to express the opinion that dwarf-red Acala represents a distinctly new type of red-plant coloration in upland cotton.

The association between dwarfing and red-plant color is apparently complete; in over 1,200 homozygous red plants no exception to this rule was found. In the heterozygous condition both the color and height of plant are uniformly intermediate between the dwarf-red and normal green plants. This evident association between red-plant and dwarfism suggests linkage or pleiotropism. To date, few cases of linkage of this sort of association have been authenticated in cotton.

Chlorophyll determinations of the leaves of dwarf-red Acala, normal green Acala, as well as several other varieties of red-plant cotton, show that all have a very similar chlorophyll content. Other varieties of red-plant cotton have grown to normal height in the station nursery. This observation would largely dispel any conclusion that dwarfing was due to any local conditions of soil or climate.

INHERITANCE OF THE DWARF-RED CHARACTER IN THE CROSS DWARF-RED×NORMAL GREEN ACALA

Homozygous dwarf-red Acala when crossed with normal green produced in the F_1 generation a progeny that was uniformly intermediate in both color and height of plant between the two parental types. As mentioned above, the "crinkled dwarfs" reported by Harland (*4*, *5*) and Hutchinson and Ghose (*9*) were entirely recessive in the F_1 or heterozygous condition. However, the intermediate red coloration in the dwarf-red heterozygote agrees with such studies previously reported (*1*, *6*, *8*, *14*, *15*, *17*). The F_2 generation segregated into the monohybrid ratio of 1:2:1. Table 2 presents the data of this segregation along with χ^2 for goodness of fit to a 1:2:1 ratio. Corresponding P values indicate a satisfactory agreement between the theoretical 1:2:1 ratio and observation.

TABLE 2.—*Segregation of the F_2 generation in the cross between dwarf-red Acala and normal green Acala*

Progeny	Dwarf red	Intermediate	Normal green	χ^2	P
93	88	155	99	3.70	0.20–
10-20	6	12	6	.00	1.00
10-21	6	15	3	2.25	.30+
21-1	19	28	19	1.52	.50–
21-11	28	47	26	.56	.80–
20-15	14	32	14	.27	.90–
20-22	18	42	17	.66	.70+
20-24	20	32	16	.71	.70+
20-1	20	41	20	.01	.99+
20-10	18	38	15	.61	.70+
20-13	36	44	23	5.47	.05+
6-2	8	16	9	.09	.95+
Total	281	502	267		

A backcross of the heterozygous dwarf-red to either parental type produces in the following generation a monohybrid ratio of 1 heterozygote to 1 parental type. Table 3 reports the results of the backcross between a heterozygous dwarf-red and normal green. Chi-square and the corresponding P values indicate a very satisfactory agreement between the observed and the theoretical 1 : 1 ratio. The backcross to the other parental type, dwarf-red, resulted in 23 intermediate red plants and 25 normal dwarf-red plants. As will be readily seen, this also is a very satisfactory agreement with the theoretical 1 : 1 ratio.

TABLE 3.—*Segregation of the backcross generation in the cross between the F_1 and the normal green Acala*

Progeny	Intermediate red	Normal green	χ^2	P
3-2	26	32	0.62	0.50–
4-2	17	16	.03	.90–
51	13	10	.39	.50+
40	9	7	.25	.70–
Total	65	65		

SUMMARY

The mutation of Acala cotton herein designated as "dwarf-red" originated as a chimera on an otherwise normal green plant.

Seeds produced by the chimera were heterozygous for the dwarf-red character.

A new type of red-plant color in upland cotton is described.

A new type of dwarfing in cotton is described which is not recessive in the heterozygous condition.

Dwarfing in this type is closely associated with red-plant color. Possibly both expressions are controlled by the same factor.

Dwarf-red Acala when crossed with normal green Acala produced an F_2 generation that was intermediate in both color and plant height between the parental types.

The F_2 generation of the cross between dwarf-red Acala and normal green Acala segregated into the 1:2:1 ratio, inducing a simple monohybrid.

Likewise the backcross generations obtained from the crosses made between the F_1 or the heterozygous dwarf-red and the parental

types verified the monohybrid condition, both segregating into the 1:1 ratio.

Indications are that dwarf-red is controlled by a single factor.

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U. S. GOVERNMENT PRINTING OFFICE: 1942